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REMARKS

Claims 1-20 were pending in this application. Claims 2, 4, and 11-12 were withdrawn from consideration by the Examiner as being drawn to a non-elected invention. Claims 2, 4, 11-12, and 19-20 are canceled herein. Applicants expressly reserve their right to pursue claims to the subject matter of these claims in related applications. Claims 1, 3, 5-10, and 13-18 are amended herein. Support for the amendments is found throughout the specification as filed, *e.g.*, ¶1, ¶21, and ¶38 of the substitute specification. Claims 1, 3, 5-10 and 13-18 are currently pending. No claim has been allowed.

Formal Matters

Applicants gratefully acknowledge the withdrawal of the objection to the specification in view of the substitute specification submitted.

Applicants also gratefully acknowledge the withdrawal of the rejections under 35 U.S.C. § 112, first paragraph, as well as the rejections under 35 U.S.C. §§ 102 (b) and 103 (a).

Applicants include herein a courtesy copy of U.S. Patent No. 5,424,398 (Middeldorp et al.) as Exhibit A.

Applicants note that the Examiner has requested the identification of other co-pending applications and/or issued patents that are related or might be interfering with the instant application. *See* Action mailed December 1, 2003 at page 9. Applicants note that an interference analysis is not required of an applicant. Nonetheless, Applicants are aware of no pertinent co-pending application and/or issue patents that are related or might be interfering with the instant application. Applicants do note that there are two additional published applications, *i.e.*, U.S. Application Serial No. 2003/0096225 and 2003/0096226, that relate to selecting a mutant of an antibody by contacting a phage display library of mutants of the antibody with a cell comprising the epitope recognized by the antibody.

Summary of Examiner Interview

On April 7, 2004, the undersigned participated in an interview with Examiner Wessendorf, Supervisory Patent Examiner Wang, and Biotechnology Specialist Caputa. The undersigned greatly appreciate the time and effort expended by all of the parties and believe that the interview was very productive. The interview can be summarized as follows: With regards to the outstanding rejections under 35 U.S.C. § 112, second paragraph, Mr. Caputa indicated that these rejections were overcome if the proposed amendments to the claims were made of record. The rejection under the judicially-created doctrine of obviousness-type double patenting was not discussed because a copy of the Middeldorp reference was unavailable for review by any of the Examiners. The undersigned agreed to provide a copy of this reference and relevant arguments with the instant response so that all parties can appropriately review the outstanding rejection. *See* Exhibit A. The parties agreed that the proposed amended claims drawn to the use of a heterologous set of oligopeptides for contacting a library of polypeptides are not anticipated by Burnie et al. (*Infect. Immun.* 1996). Examiner Wessendorf indicated that she would perform another search once the revised claims were made of record.

Objection Under 35 U.S.C. § 132

According to the Action, the amendment filed September 2, 2003 contains new matter and is therefore objected to under 35 U.S.C. § 132. The Examiner asserts that the term "or" at page 1, line 6 is not supported by the original disclosure. The term "or" is deleted herein.

In view of the above, Applicants respectfully submit that the objection may be withdrawn.

Objection to the Specification

Claim 3 was objected to because allegedly it is not positively disclosed in the specification. The specification is amended herein to positively disclose claim 3 as currently amended. Therefore, this objection may be withdrawn.

Rejection under 35 U.S.C. §112, Second Paragraph

Claims 1, 3, 5-10 and 13-20 were rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner asserts that the terms "peptide" and "proteinaceous target" are confusing in that they go against the conventional usage in the art. The Examiner also argues that there is nothing in the specification that defines a set as a multiplicity of oligopeptides. According to the Examiner, Applicants have not responded to the rejection of claims 7 and 15 as confusing and broadening the base claim, and therefore the Examiner believes that Applicants have acquiesced to the rejection. The Examiner also alleges that the single chain fragment in claim 8 is the same as the scFv recited in claim 9. The Examiner further asserts that in the absence of any additional step, claim 1 is a duplicate of claim 3. Applicants respectfully traverse these rejections.

Applicants respectfully submit that the meaning of the term "peptide" is sufficiently clear to one of skill in the art to inform the public of the boundaries of metes and bounds of the claimed invention and therefore each term fulfills the requirement for clarity under 35 U.S.C. § 112, second paragraph. *See* MPEP § 2173.05 (a) ("Applicants ... are required to make clear and precise the terms that are used to define the invention whereby the metes and bounds of the claimed invention can be ascertained."). Nonetheless, in an effort to expedite the prosecution, the claims are amended herein to recite a "polypeptide".

Similarly, Applicants submit that the meaning of the term "proteinaceous target" is readily apparent in view of the specification and what is known in the art. However, the claims are amended herein in an effort to expedite the prosecution of this application to recite target "protein", thereby rendering this rejection moot.

While Applicants believe the term "a set" is fully supported in the specification as a multiplicity of oligopeptides, the claims are amended herein to further clarify that the claimed set of oligopeptides are heterologous. The support for this interpretation of "set of oligopeptides" is found throughout the specification. For example, the instant specification refer to sets of oligopeptides representing (sub)regions of the target molecule. *See* the substitute specification at ¶38. As these sets of oligopeptides represent different subregions or regions of the target molecule, they are by definition heterologous. The specification further indicates that the oligopeptides can be "overlapping or nonoverlapping sets of multimers" from the target protein. *Id.* Such language clearly discloses the use of sets of oligopeptides that represent different portions of the target protein, and thus are heterologous to one another. This is further supported by the embodiment disclosed in Example 1 beginning at ¶38 of the specification. In this Example, Applicants disclose the synthesis of a set of 25 non-overlapping oligopeptides (12mers) that span the extracellular domain of the CD64 molecule. Again, because these oligopeptides span the entire domain of a single molecule, they are heterologous to one another. Applicants also note that the claimed heterologous oligopeptides also include peptides that differ by even a single amino acid. *See, e.g.*, the specification at ¶21. Therefore, the recitation of a "set of heterologous oligopeptides" is sufficiently precise to convey to the ordinary artisan the scope of the claimed methods in view of what is known in the art and what is disclosed in the instant specification.

Applicants have not acquiesced to the rejection of claims 7 and 15 as confusing and broadening the base claim. The base claims, *i.e.*, Claims 1 and 3, recite the "displaying [of] the antibody or peptide on the surface of a replicable display package". Claims 7 and 15 recite one

means of displaying the antibody or peptide on the surface of a replicable display package, *i.e.*, by the insertion of a genetic sequence encoding the antibody or peptide in a gene encoding a surface protein of the phage particle. In other words, claims 7 and 15 state a specific embodiment of the claimed method of claims 1 and 3, respectively, where the antibody or peptide is expressed on the surface of the display package through the expression of the antibody or peptide's genetic sequence in a gene encoding a surface protein. Because claims 7 and 15 state a specific embodiment of the each of the claimed methods 1 and 3, respectively, neither of these dependent claims broaden the base claim. Rather, these claims are each narrower in scope in view of the claimed specific means of displaying the antibody on the phage surface. Applicants appreciate the acknowledgement of these claims as proper during the Examiner Interview.

Applicants strongly object to the Examiner's characterization of the antibodies of claims 8 and 9 as the same. A single chain antibody fragment and a ScFv are not the same. An ScFv is "a single chain molecule composed of both heavy- and light-chain variable regions fastened together by a flexible linker." *See* Exhibit B (emphasis added). A single chain antibody fragment is not limited to variable regions only and thus can include more than the variable regions of the heavy and light chains in the single chain fragment. Therefore, these antibodies are not the same.

Applicants submit that claim 1 presents no ambiguity regarding the method claimed. It is unclear what the Examiner means by a sample for the reaction and identification to occur as the method does not require a sample *per se*. The methods of the instant claims uses a phage library and a set of heterologous oligopeptides. Thus, it is not believed that such claim language is appropriate. Furthermore, Applicants believe that claim 1 is not a duplicate of claim 3. On the one hand, claim 1 requires only the characterization of the bound antibody. Claim 3, on the other hand, includes the characterization of the unbound phage antibody as well as the bound phage antibody. Therefore, claim 1 and claim 3 are not duplicates of one another.

In view of the foregoing, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, second paragraph.

Rejection Under the Judicially Created Doctrine of Obviousness-Type Double Patenting

Claims 1, 3, 5-10 and 13-20 were rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1, 5, and 6 of U.S. Patent No. 6,265,150 (the '150 patent) or claims 1, 5 and 6 of U.S. 20002/013228 ('228 application) in view of Middeldorp *et al.*, U.S. Patent No. 5,424,398. According to the Examiner, each of the cited patents discloses the instant method as claimed except for the use of a set of oligopeptides as the target antigen. The Examiner asserts that Middeldorp cures this deficiency in its disclosure of the synthesis of individual antigens or oligopeptides on a solid support using the Geysen method. Applicants traverse this rejection.

The '150 patent or the '228 application in view of Middeldorp fail to render the claimed methods obvious because the combination of reference fails to teach a method using a library of polypeptides displayed on the surfaces of replicable display packages that is then contacted with a set of heterologous oligopeptides derived from a target protein to identify polypeptides capable of binding to the target protein and/or oligopeptides thereof. Thus, the combination of cited references lacks each and every element of the claimed invention. First, the claims of the '150 patent and the '228 patent are drawn to a method of obtaining phage antibodies directed to a cell surface antigen using whole cells. A person of ordinary skill in the art would not conclude that the use of whole cells is an obvious variation of the use of a set of heterologous oligopeptides in a method to identify phage antibodies that bind certain antigens. Whole cells differ from oligopeptides in size, charge, shape, and in some cases, binding affinity. Methods employing whole cells would require distinct binding conditions relative to methods employing oligopeptides. For example, buffers, temperature, incubation times, suitable detection methods, and assay set up are likely to be different.

Furthermore, the intended purpose of the methods of the '150 patent and the '228 application are to identify antibodies which bind cell surface antigens on intact cells. The use of oligopeptides to identify binding antibodies is not restricted to surface antigen as targets, and therefore represents a change in the principle of operation from the claimed methods of '150 patent and the '228 application. *See* MPEP § 2143.01 ("If the proposed modification ... of the prior art would change the principle of operation of the prior art invention being modified, then the teaching of the references are not sufficient to render the claims prima facie obvious." (citation omitted)).

Second, Middeldorp fails to cure this deficiency. Like Burnie (addressed below), Middeldorp employs the Geysen method to identify an immunodominant epitope of a target antigen. In other words, Middeldorp employs oligopeptides sets for use with sera from antigen-reactive patients to identify the particular peptide that is immunodominant, *i.e.*, bound by the antibodies of most individuals exposed to the target antigen. None of the cited references provide a method to simultaneously and specifically identify the bound oligopeptide and the bound polypeptide. In the methods of '150 patent and '228 application, the antibody can be identified by isolating and growing out the phage antibody. However, this provides no indication of the identity of the peptide bound by the phage antibody. Middeldorp provides a method of identifying the peptide bound by antibodies in the sera of immunoreactive patients without providing any mechanism for identifying the specific antibody that binds the specific oligopeptide. Therefore, neither the '150 patent in view of Middeldorp nor the '228 application in view of Middeldorp teach each and every element of the claimed methods. In the absence of such disclosure, the instant methods are patentably distinct from the claimed methods of the '150 patent and the '228 application.

In view of the above, Applicants respectfully submit that the rejection under the judicially-created doctrine of obviousness-type double patenting may be withdrawn.

Rejection Under 35 U.S.C. § 102 (a)

Claims 1, 3, 5-10, and 13-20 are rejected under 35 U.S.C. § 102 (a) as being anticipated by Burnie et al. (*Infect. Immun.* 1996) for reasons of record. The Examiner maintains that Burnie discloses the reacting of phage antibody with overlapping peptides prior to panning and cites to the paragraph in Burnie bridging pages 1600 and 1601. Applicants traverse this rejection.

Applicants respectfully submit that Burnie fails to teach the reacting of phage antibody with overlapping peptides prior to panning as asserted by the Examiner, and therefore fails to teach each and every element of the claimed methods. A careful reading of the entire reference and the supporting references unambiguously demonstrates that no panning of the phage antibodies with overlapping oligopeptides is performed or contemplated in Burnie. Simply stated, Burnie discloses a method using two separate steps to identify scFv phage antibodies specific for a target antigen. In the first step, the antigen is epitope mapped using the Geysen method. Once the epitopes are defined using the sera, the epitope-encoding peptides, *i.e.*, individual peptides, are used to pan the scFv phage antibodies in the second step. Thus, contrary to the Examiner's assertion, the scFv phage antibodies are never panned against a set of overlapping oligopeptides.

Applicants submit that the Geysen method is, quite simply, a method to identify meaningful antigenic epitopes, and nothing more. *See* Exhibit C. It involves constructing a series of overlapping peptides on pins. These pin-bound oligopeptides are then incubated with sera. After the incubation, the unbound antibody is washed away, and a conjugate containing solution is added. The conjugate binds the bound antibody, and thereby permits the detection of the bound antibody. The bound antibodies are read in an ELISA format. Because each of the oligopeptides is bound to a pin at an assigned position, the presence of the detectable label at that position indicates that an antibody binds that oligopeptide. Thus, if a number of immune seras have antibodies that bind at a particular oligopeptide, it is likely that this oligopeptide represents an immunodominant epitope for the antigen. Thus, this method facilitates epitope mapping of an antigen using various immune and

non-immune sera. The use of the Geysen method to map immunogenic epitopes in a bacterial antigen is illustrated in the two references cited by Burnie. *See* Exhibits D and E.

Thus, the panning in the Geysen method is not equivalent to the panning in the instant methods. In particular, the Geysen method does not employ or suggest the use of phage antibodies. Moreover, the Geysen method lacks a means to identify the antibody that binds a particular oligopeptide or any disclosure that such a means is necessary or desirable. Alternatively stated, the Geysen method is a screening method for epitopes, not for the antibodies that bind those epitopes. Geysen employs antibody binding to identify the epitopes seen in an immune response. Using oligopeptides at the tip of the pins at a concentration of 30-50 nmol, Geysen discloses the detection of antibodies bound to individual peptides via the detectable conjugate. *See* Burnie, at page 262, second column. Given the nanomolar concentration of oligopeptide used, one of ordinary skill in the art could not successfully elute off enough bound antibody to characterize it, much less readily create useful quantities of it, as is possible with the instant methods. In other words, if the bound antibody were to be successfully eluted from the oligopeptide, it would be an insufficient to achieve the intended purpose of the claimed methods, *i.e.*, to identify an antibody by its capability to bind an oligopeptide.

Finally, Burnie states unequivocally that single peptides were used to pan the phage display library after using the Geysen method to identify the epitope. At pages 1602-03, in the section entitled "Epitope mapping", Burnie describes panning sera from various patients against a set of overlapping oligopeptides. *See also* Burnie, at Table 4. Burnie selected three peptides from conserved regions in the antigen to use to generate recombinant antibodies using phage display antibodies. *See* Burnie, at page 1603, section entitled "Human recombinant antibodies." Burnie describes a highly heterogeneous family that has an identifiable fingerprint after panning with one peptide. More specifically, Burnie states:

> BstNI fingerprints of the PCR-amplified scFv inserts before panning showed a highly heterogeneous library (Fig. 5). After panning against peptide 1, one BstNI fingerprint predominated ...

Burnie, at page 1603 (emphasis added). Burnie continues describing the panning of the phage antibody library with individual peptides, peptides 2 and 3, in two separate analyses. Thus, Burnie fails to teach each and every element of the claimed methods, thereby failing to anticipate the methods at issue. Applicants appreciate the acknowledgement in the Examiner Interview that Burnie is not an anticipatory reference of the method as claimed.

In view of the above, Applicants respectfully submit that the rejection under 35 U.S.C. § 102 (a) may be withdrawn.

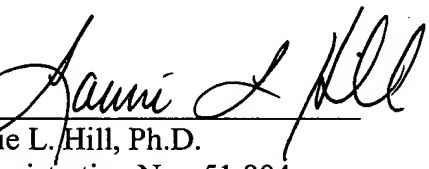
CONCLUSION

Applicants believe that all issues raised in the Office Action have been properly addressed in this response. Accordingly, reconsideration and allowance of the pending claims is respectfully requested. If the Examiner feels that a telephone interview would serve to facilitate resolution of any outstanding issues, the Examiner is encouraged to contact Applicants' representative at the telephone number below.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, Applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 313632000600.

Dated: April 21, 2004

Respectfully submitted,



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tuberculosis. Patients may develop fever, polyarthritis, erythema nodosum, and iritis. They also may experience loss of weight, anorexia, weakness, fever, sweats, nonproductive cough, and increasing dyspnea on exertion. Pulmonary symptoms occur in greater than 90% of the patients. Angiotensin-converting enzyme is increased in the serum of sarcoid patients. Disease activity is monitored by measuring the level of this enzyme in the serum. The subcutaneous inoculation of sarcoidosis lymph-node extracts into patients diagnosed with sarcoidosis leads to a granulomatous reaction in the skin 3 to 4 weeks after inoculation, which was used in the past as a diagnostic test of questionable value termed the Kveim reaction. Sarcoidosis symptoms can be treated with corticosteroids, but only in patients where disease progression occurs. It is a relatively mild disease, with 80% resolving spontaneously and only 5% dying of complications. Evidence has been provided for oligoclonal expansion of αβ T cell subsets and predominant expression of type 1 cytokines — interferon-γ (IFN-γ) and interleukin-2 (IL-12) — at sites of inflammation, suggesting that sarcoidosis is an antigen-driven, T_H1-mediated immune disorder.

sarcoma

Tumors arising from connective tissue.

Sca-1

Abbreviation for stem cell antigen 1.

SCABs (single-chain antigen-binding proteins)

Polypeptides that join the light-chain variable sequence of an antibody to the heavy-chain variable sequence of the antibody. All monoclonal antibodies are potential sources of SCABs. They are smaller and less immunogenic than the intact heavy chains with immunogenic constant regions. Among their many possible uses are in imaging and treatment of cancer, in cardiovascular disease, as biosensors, and for chemical separations.

scarlet fever

A condition associated with production of erythrogenic toxin by group A hemolytic streptococci associated with pharyngitis. Patients develop a strawberry-red tongue and generalized erythematous blanching areas that do not occur on the palms, the soles of the feet, or in the mouth. Patients may also develop Pastia's lines, which are petechiae in a linear pattern.

Scatchard analysis

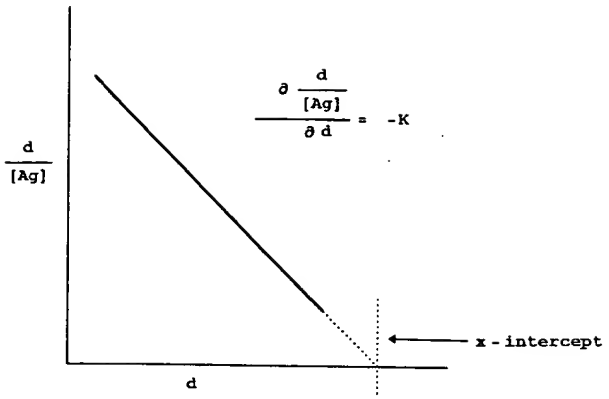
A mathematical analytical method to determine the affinity and valence of a receptor-ligand interaction in equilibrium binding.

Scatchard equation

In immunology, an expression of the union of a univalent ligand with an antibody molecule ($r/c = Kn - Kr$). To obtain the average number of ligand molecules that an antibody molecule may bind at equilibrium, the bound ligand molar concentration is divided by the antibody molar concentration. This is designated as r. The free ligand molar concentration is represented by c; antibody valence, by n; and the association constant, by K.

Scatchard plot

A graphic representation of binding data obtained by plotting r/c against r (refer to the Scatchard equation). The purpose of this plot is to determine intrinsic association constants and to ascertain how many noninteracting



Scatchard plot.

binding sites each molecule contains. A straight line with a slope of $-K$ indicates that all the binding sites are the same and are independent. The plot should also intercept on the r axis of n. A nonlinear plot signifies that the binding sites are not the same and are not independent. The degree to which the sites are occupied is reflected by the slope ($-K$). An average association constant for ligand binding to heterogeneous antibodies is the reciprocal of the amount of free ligand needed for half saturation of antibody sites.

scavenger receptors

Structures on macrophages and other cell types that bind a variety of ligands and delete them from the blood. Scavenger receptors are especially abundant on Kupffer's cells of the liver.

SCF (stem cell factor)

A substance that promotes growth of hematopoietic precursor cells and is encoded by the murine SI gene. It serves as a ligand for the tyrosine kinase receptor family protooncogene termed c-*kit*. It apparently has a role in embryogenesis in cells linked to migratory patterns of hematopoietic stem cells, melanoblasts, and germ cells.

ScFv

A single-chain molecule composed of both heavy- and light-chain variable regions fastened together by a flexible linker.

Schick, Bela (1877–1967)

Austro-Hungarian pediatrician whose work with von Pirquet resulted in the discovery and description of serum sickness. He developed the test for diphtheria that bears his name. (Refer to *Die Serumkrankheit* [with Pirquet], 1905.)

Schick test

A test for susceptibility to diphtheria. Standardized diphtheria toxin is adjusted to contain 1/50 MLD in 0.1 mL, which is injected intracutaneously into the subject's forearm. Development of redness and induration within 24 to 36 hours after administration constitutes a positive test if the condition persists for 4 days or longer. The presence of 1/500 to 1/250 or more of a unit of antitoxin per milliliter of the patient's blood will result in a negative reaction because of neutralization of the injected toxin. Neither redness nor induration appears if the test is negative. An individual with a negative test possesses

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Strategies for epitope analysis using peptide synthesis

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(Received 9 March 1987, revised received 11 May 1987, accepted 13 May 1987)

A recently developed approach to the synthesis and ELISA screening of large numbers of peptides is described. The method has created the opportunity to tackle questions about the sites and specificity of antigenic determinants which were formerly thought to be too difficult to answer. The various strategies for application of this method are described along with examples of their successful use. They include a procedure for locating all the continuous antigenic peptides of a protein antigen, and the identification of non-replaceable amino acid residues within an antigenic peptide. An approach to the determination of amino acid residues involved in the epitope for any monoclonal antibody is also described. These strategies open up the prospect of rapid mapping of the antigenic properties of hitherto poorly understood antigens.

Key words: Peptide synthesis; ELISA; Epitope; Mimotope

Introduction

Solid-phase methods for the synthesis of peptides (Merrifield, 1963) have proven to be extremely versatile tools for the study of protein-protein interactions. Until the advent of solid-phase peptide synthesis, investigation of the sites and nature of the epitopes of protein antigens proceeded slowly, using the laborious methods of testing protein cleavage fragments and chemical modification of proteins. Synthetic peptides are now extensively used in the fields of immunology, hormone-receptor interactions and vaccine research. The general approach is still to synthesize peptide homologs or analogs of sections of a larger polypeptide or protein and evaluate their biologically relevant activity. Because there have been limitations on the number of peptides which could be readily synthesized and tested, the choice of peptides has often been made on the basis of predictive algorithms which have appeared in the scientific literature in recent years (Hopp and Woods, 1981; Fraga, 1982; Westhof et al., 1984; Fanning et al., 1986; Novotny et al., 1986; Thornton et al., 1986). Clearly, for these strategies to be successful there must be a good correlation between the predictor parameters and the desired biological activity.

Limitations on the systematic use of peptides have included the cost and magnitude of the facilities required to provide a significant number of peptides for evaluation. In addition, peptides are frequently coupled to a solid surface or a carrier molecule in order to allow detection of binding interactions between the peptide and a protein.

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Abbreviations: ABTS, 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid); BOC, *t*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane, DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HOBT, 1-hydroxybenzotriazole; MAb, monoclonal antibody; OPD, *o*-phenylenediamine; PBS, phosphate-buffered saline; swMb, sperm whale myoglobin.

This coupling procedure can significantly affect the presentation of the peptide, even to the extent of masking potentially reactive amino acid side chains.

We have demonstrated (Geysen et al., 1984) that by relaxing the criteria for quantity and purity, peptides can be synthesized in a re-usable format in numbers three orders of magnitude greater than by conventional means. A systematic approach involving complete sets of related peptides is therefore possible. Results obtained from such a systematic approach are independent of the accuracy of any predictive algorithm, and are providing a comprehensive database from which more accurate relationships can be formulated (Geysen et al., 1985b, 1987). An additional advantage of this systematic approach is that the number of negative controls incorporated into any experiment is very large, providing a statistical basis to set criteria for a significant positive interaction.

Improvements in the synthesis procedures, and experience gained by their use, over the past 3 years have led to a number of defined strategies which are useful in studying antigen-antibody interactions. During this period more than 200 000 peptides have been synthesized and tested with a variety of both monoclonal and polyclonal sera to yield a database of greater than 1 000 000 assay values. Various antigens have been studied, from small protein antigens such as myoglobin and myohemerythrin whose tertiary structure is known from X-ray crystallography (Rodda et al., 1986a; Geysen et al., 1987) to complex antigens of unknown tertiary structure such as foot and mouth disease virus capsid protein (Geysen et al., 1984, 1985). The results of these studies have indicated that previous concepts of antigenicity of relatively simple antigens (Atassi, 1984) need extensive revision.

The current methodology requires only basic skills in organic chemistry, and can be used to synthesize more than 2000 peptides (hexapeptides) per 10 working days. Because peptides are produced in a re-usable format, the limitation to data collection very quickly devolves to the testing and evaluation capability of the group. Our group presently tests about 4000 peptides each working day.

Materials and methods

Synthesis of peptides

Chemicals were of analytical reagent grade or the best available grade unless otherwise specified. Specially molded high-density polyethylene rods (diameter 4 mm, length 40 mm) were washed in toluene, dried, then treated with 10% (w/v) perchloric acid in water for 2 h. After several rinses in water, the rods were suspended in deaerated 6% (v/v) acrylic acid (BDH cat. no. 27046) in water containing 0.005 M copper sulfate. Bottles filled with rods were gamma-irradiated in a commercial sterilization plant at a dose of 0.8 Mrad (Muller-Schulte and Horster, 1982) to graft polymerize the acrylic acid to the rods as polyacrylic acid. After grafting, the rods were washed in water, 0.1 M sodium carbonate, water, 0.1 M hydrochloric acid, and finally with water again before thorough drying at 60 °C overnight. These dried, grafted rods were assembled into specially molded polyethylene holders designed to hold 96 rods in the format and spacing of a microtiter plate. Subsequent reactions at the tips of the rods were carried out in wells drilled in a teflon block in an 8 × 12 matrix to match the rod spacing.

A *t*-butyloxycarbonyl (BOC)-protected amino group was introduced by reacting the polyacrylic acid on the rods for 2–3 days with 0.15 ml per rod of a mixture containing 78 mM dicyclohexylcarbodiimide (DCC, Fluka cat. no. 36650), 142 mM hydroxybenzotriazole (HOBT, Fluka cat. no. 54802), 72 mM mono-*t*-butyloxycarbonyl-1,6-diaminohexane hydrochloride (Fluka cat. no. 15392) and 72 mM triethylamine in dimethylformamide (DMF). BOC deprotection was effected using a conventional trifluoracetic acid treatment (Table I). For each step, rods in holders were placed in a bath of reagent, which was covered during the specified incubation time. After the trifluoroacetic acid treatment, the rods were allowed to dry in air prior to neutralization. The potential problems of exothermic reactions and precipitation of salt were avoided by this precaution, and also by reason of the small amount of amine on the rods, its surface location on the tips of the rods, the high solubility of the salt in ethanol, and the large volume (> 200 ml) of solvent present in each bath.

A purposely limited amount of BOC-β-alanine

was coupled to the free amino groups by reacting each rod for 4 h with 0.15 ml of DMF containing 30 mM BOC-β-alanine, 33 mM DCC and 60 mM HOBT, to complete a peptide-like spacer. Unreacted amino groups were acetylated by reaction for 1 h with 0.165 ml per rod of a DMF : acetic anhydride : triethylamine mixture (5 : 2 : 1 v/v/v). Kits containing rods prepared to this stage are available from Cambridge Research Biochemicals, Cambridge, U.K. Rods were then BOC-deprotected (Table I) and the first amino acid coupled, as dictated by the sequence to be synthesized. DCC/HOBT-mediated coupling reactions were carried out overnight in 0.15 ml per rod of a DMF solution of the particular side-chain protected BOC-amino acid. Concentrations of reactants were as for BOC-β-alanine (above).

The following side-chain protecting groups were used: *O*-benzyl for threonine, serine, aspartic acid, glutamic acid and tyrosine; carbobenzoxy for lysine; tosyl or 4-methoxybenzesulfonyl for arginine, tosyl for histidine, and 4-methoxybenzyl for cysteine. All protected amino acids were from either Bachem (Switzerland) or the Peptide Institute (Japan).

The synthesis of the required peptides was completed by successive cycles of BOC deprotection (Table I) and addition of one amino acid per rod per day as for the first amino acid (above). A microcomputer program was used to direct the addition of the correct amino acid to each rod on each day. At the completion of the final coupling reaction, and after removal of the BOC protecting group, the terminal amino group was acetylated as described for rods after the 4 h coupling of BOC-β-alanine (above).

Side-chain deprotection was achieved by treatment with 0.5 ml per rod of 50 mM boron tris(trifluoracetate) (Merck cat. no. 818067) in dry trifluoroacetic acid for 90 min at 4° C, with particular care taken to exclude moisture (Pless and Bauer, 1973; Bauer and Pless, 1976). Parallel deprotection of BOC-L-arginine(tosyl) and evaluation by thin-layer chromatography confirmed the effectiveness of the boron tris(trifluoracetate) method for quantitative removal of the tosyl group, the most stable side-chain protecting group used in this study (unpublished work). Additional evidence for the effectiveness of the side chain deprotection method was derived from ELISA testing of rods bearing sequence GDLGSIA, a characterized epitope recognized by both polyclonal and monoclonal antibodies (Geysen et al., 1985). Rods which had been treated with boron tris(trifluoracetate) as above gave a titer (endpoint optical density = 1.0) of > 100000, whereas rods which had not been so treated gave a titer of < 200.

Before testing by ELISA, rod-coupled peptides were washed in aqueous 0.1 M sodium phosphate, pH 7.2, for 30 min, then subjected to a cleaning or 'disruption' process as follows. Rods were immersed in an aqueous solution, prewarmed to 60° C, containing 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.1 M sodium phosphate, pH 7.2. While in this solution, they were sonicated for 10 min at a power input of about 1000 W for a bath volume of 5 liters. Rods were then immersed in water at 60° C, followed by boiling methanol, and air dried.

ELISA

ELISA reactions on the rods were carried out with serum and conjugate solutions in polystyrene microtiter trays. The tips of the rods, with the synthesized peptides still bound to them, were immersed for 1 h at 25° C in 0.2 ml of a 'diluting' buffer comprising 1% bovine serum albumin (Fraction V), 1% ovalbumin (Sigma cat. no. A5253) and 0.1% Tween 20 (ICI) in 0.85% saline buffered with 0.01 M phosphate, pH 7.2, to block nonspecific adsorption of antibodies. A suitable dilution of antiserum was prepared in the diluting buffer and the rods were incubated in 0.175 ml overnight at 4° C with agitation. Rods were washed four times in baths containing 0.05% Tween 20 in phosphate-buffered saline, pH 7.2 (PBS/Tween) for 10 min each time, to remove unbound antibody. Bound antibody was detected by reaction for 1 h at 25° C in 0.175 ml 'conjugate', comprising a saturating level (0.25 mg/l) of horseradish peroxidase-labelled goat anti-IgG, specific for the species under test (Kirkegaard and Perry Labs, Maryland) made up in diluting buffer. The rods were again washed four times in baths of PBS/Tween, to remove excess conjugate, and the presence of enzyme was detected by reaction for 45 min at 25° C with 0.15 ml of a freshly prepared enzyme substrate solution. In earlier work this

TABLE I

BOC-DEPROTECTION CYCLE

Reagent	no. of baths	Time/bath
Ethanol	1	15 s
Dichloromethane (DCM)	3	5 min
50% trifluoroacetic acid in DCM	1	30 min
10% triethylamine in boiling ethanol	1	15 s
DCM	1	5 min
10% triethylamine in DCM	2	5 min
DCM	3	5 min

consisted of 40 mg of *o*-phenylenediamine (OPD) and 0.018 ml of '120 vol' hydrogen peroxide in 100 ml of 0.1 M phosphate/citrate buffer, pH 5.0. In later work, this was changed to 50 mg of ABTS (Boehringer Mannheim, cat. no. 122661) and 0.03 ml of '120 vol' hydrogen peroxide in 100 ml of 0.1 M phosphate/0.08 M citrate buffer, pH 4.0. Color development was stopped simultaneously in all wells by removing the rods from the microtiter plate containing the substrate. For OPD, the colored product was measured in a Titertek Multiskan MC plate reader in the dual wavelength mode at 450 nm against the reference wavelength of 690 nm. For ABTS, the measurement was performed at 405 nm against the reference wavelength of 492 nm. ELISA absorbance values were transmitted to a microcomputer and stored on diskette for later analysis.

To remove bound antibody from rods prior to retesting of the peptides, rods were disrupted as described above and could be stored in the dry state if not required immediately. Control ELISA tests, in which no antiserum was present in the diluting buffer during the overnight incubation step, showed that this disruption procedure removed all detectable antibody. Amino acid analysis also confirmed the absence of amino acids other than those contributed by the peptide synthesized on the rod.

Interpretation of data

The choice of the small-scale, surface synthesis format for peptide synthesis was based on the following reasoning:

(1) In order to detect by ELISA antibodies able to bind a particular peptide, the quantity of peptide need only be in the range of pmol (Bittle et al. 1982). Since the level of peptide on the tips of the rods, as determined by amino acid analysis (see below) is typically 30–50 nmol, there is more than sufficient peptide for detection by ELISA. The absorbance obtained in an ELISA for a given peptide is therefore expected to depend mainly on the concentration of the antibody population with the correct specificity for reaction. The difference in the absorbance obtained with peptides synthesized with densities varying over two orders of magnitude is similar to the 10–30% variation observed between replicate syntheses (unpublished data). Thus the absorbance is essentially independent of the peptide density, expressed as reacting groups/mm^2 of support, over the range used in this work.

(2) High purity for the peptide used in the detection of antibodies is not a necessary condition. The majority of serological tests rely on the specificity of antibodies to detect a given antigen in the presence of large amounts of irrelevant protein. In testing rod-coupled peptides using hyperimmune sera or monoclonal antibodies (MAbs), we rely on the selectivity of the antibodies to ensure that the binding observed was due to reaction with the nominal sequence synthesized and not with the small amounts of deletion peptides, termination peptides or other by-products which inevitably form during peptide synthesis. Where peptides were identified as binding an antibody and were subsequently resynthesized and purified in mg quantities, the pure peptide when coupled to rods also bound the antibody, confirming the original result. It must be emphasized that the use of surface-synthesized peptides as described here is a screening procedure leading to the identification of sequences for further investigation on a larger scale, i.e., on a scale which allows full characterization of the reacting peptide. Because of the uncertainty of the final purity of the peptide on any given rod, the method suffers from the disadvantage that a negative result (no binding) cannot be taken as proof of the absence of antibody able to bind to that nominal sequence.

(3) Control peptides: The rod format of peptide synthesis provides the opportunity to synthesize many peptides simultaneously. In many of the

systematic syntheses reported below, a large number of the peptides effectively act as negative controls in the test. When peptides on adjacent rods differ in sequence by only one or two amino acids at their termini, the observation of antibody binding to one rod but not to adjacent rods is particularly good evidence for the specificity of the test. The background level can thus be defined as the mean absorbance for the set of peptides giving a uniformly low value in the test.

Assessment of peptide synthesis

The quality of the peptides was assessed by:

Amino acid analysis. Whole rods or the tips of rods on which peptide had been synthesized were subjected to hydrolysis in constant-boiling hydrochloric acid in a nitrogen-purged, evacuated ampoule at 110 °C for 24 h. The rod was removed, the solution evaporated to dryness and the residue reconstituted in amino acid analysis buffer A for analysis on a Waters ion exchange amino acid analyzer using pH gradient elution, post-column *o*-phthalaldehyde derivatization and fluorescence detection. Samples were compared with standards (Pierce cat. no. 20088) for calculation of absolute levels of amino acid. Accuracy was probably lower than for conventional amino acid analysis because of the presence of an excess of hydrolysis products, such as hexamethylene diamine and polyacrylic acid. Tryptophan was not determined as it was destroyed by this sample preparation method. Cysteine and methionine were detected only qualitatively, as cysteic acid and methionine sulfoxide, respectively. Proline was determined on separate analyzer runs following treatment of the sample with sodium hypochlorite.

Synthesis efficiency. Control peptides included in each synthesis were designed to check the coupling of each amino acid on each day of synthesis. This approach required the synthesis of 20 control peptides. To simplify the subsequent amino acid analysis, sequences were synthesized in which no amino acid was repeated, and in which the analysis would yield unequivocal results, e.g., Glu (E) and Gln (Q) could not be included in the same peptide. It was found that the above conditions were met in peptides up to 9 residues in length by synthesizing peptides with sequences determined by the alphabetical order of the single letter code for each amino acid (IUPAC-IUB, 1968). Thus, for a hexapeptide synthesis, sequences GFEDCA, HGFEDC, IHGFED,..., EDCAYW, FEDCAY were synthesized as controls for amino acid analysis. Table II presents the results of the amino acid analysis of a set of typical control peptides. It can be seen that, with two exceptions, acceptable levels of incorporation of amino acids were achieved. Failure to detect methionine on the day 6 control may have been due to the difficulty of getting satisfactory recovery of this amino acid by this

TABLE II

AMINO ACID ANALYSIS OF CONTROL PEPTIDES

Day	Amino acid [a]																		
	A	C	D	E	F	G	H	I	K	L	M	N	Q	R	S	T	V	Y	Mean
1	1.0	D [b]	0.8	1.0	1.3	0.8	1.2	1.0	0.8	0.3	D	0.5	2.2	1.1	1.1	1.1	0.9	0.4	1.0
2	0.8	D	0.8	0.8	1.1	1.4	0.6	0.6	1.2	0.8	D	0.9	1.5	1.6	0.9	1.0	0.9	1.0	1.0
3	1.4	D	0.8	0.9	1.1	0.9	1.0	0.5	0.9	1.9	D	0.6	0.5	1.5	1.7	0.8	1.1	0.8	1.0
4	0.8	D	1.5	0.9	1.0	0.7	0.7	0.8	0.5	1.2	D	0.7	0.7	0.5	1.3	1.7	0.9	0.7	0.9
5	0.6	D	0.9	0.6	0.9	0.6	0.6	0.5	0.5	0.5	D	0.8	0.3	0.7	0.4	1.2	1.1	0.9	0.7
6	0.8	D	0.7	1.2	0.4	0.7	0.6	0.5	0.4	nd [c]	nd	0.8	0.7	0.5	0.6	0.4	1.2	1.0	0.7
Mean	0.9		0.9	0.9	1.0	0.9	0.8	0.7	0.7	0.9 [d]		0.7	1.0	1.0	1.0	1.0	1.0	0.8	0.9

[a] All values in the table are the molar ratio of the amino acid to the β-alanine on that rod treated as an internal standard. Cysteine (C) and methionine (M) are degraded to a variable extent in the hydrolysis method used. The method is unsuitable for the estimation of tryptophan. In two analyses for proline (see text), a mean ratio of 0.7 was obtained.

[b] Detected (see text).

[c] Not detected (see text).

[d] Mean of 5 days only.

method. The failure to detect leucine on the same day is of greater concern, as it may represent an operational error in activation or addition of this amino acid to the rods. Detection of a coupling failure necessitates a repeat of the synthesis to verify the results.

Control ELISA-testable peptides. A refinement introduced in the latter stages of this work was the synthesis, within each group of 96 rods on a single rod holder, of two peptides which could be checked by ELISA. One of these peptides (acetyl-PLAQ-link-rod) is known to bind specifically to an anti-sperm whale myoglobin MAb, and the other (acetyl-GLAQ-link-rod) is a similar but non-binding peptide for the same MAb (Geysen, 1985). At the conclusion of each synthesis, these rods were tested with the MAb under standardized ELISA conditions. If the expected absorbance values in the ELISA for the positive and negative peptides were obtained, this provided evidence for successful synthesis in advance of amino acid analysis results becoming available.

Stability on repeat testing

In order to evaluate the long-term stability of peptides to repeated ELISA testing, a set of overlapping peptides (tri- to heptapeptides) homologous with a portion of sperm whale myoglobin, residues 81–96 (^{81}HHEAELKPLAQSHATK^{95}) was synthesized. Four rabbit polyclonal sera and one monoclonal ascites containing antibodies reactive with some of the peptides comprising the set were repeatedly tested for a total of 50 tests, i.e., a total of ten tests with each serum. When the absolute signal obtained in the test is evaluated as a function of the test number, a gradual decrease is observed (Fig. 1). In addition, the scatter about the line of best fit is an indication of the inherent between-test variability in the assay itself. In the example shown, the useful life of the peptide set is about 50 tests, i.e., the number of tests after which the signal obtained is approximately half that obtained in the initial test.

Applying the same criterion to many other sets of test results, we have found that a set of peptides is useful for 30–60 tests. No obvious correlation between the amino acid composition of individual peptides and their useful testing lifetime could be established.

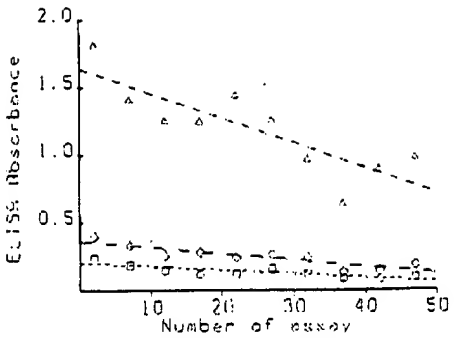

Fig. 1. Decrease in ELISA absorbance on repeat testing of rod-coupled peptides. △, peptide ^{86}PLAQ^{91}; ◇, peptide ^{86}LAQS^{91}; □, peptide ^{87}KPLA^{90}, all homologous with swMb. Every fifth assay of the peptides was with a 1/400 dilution of an anti-swMb monoclonal ascites fluid previously found to bind specifically to peptide PLAQ (Geysen, 1985; Rodda et al., in preparation) and the ELISA developed with OPD/hydrogen peroxide substrate. The lines of best fit are also shown for the three peptides.

Results

'Scanning'

Immunization of a vertebrate with a protein antigen evokes an immune response, the major soluble products of which are the immunoglobulins. These are distinguished by their specificity for the inducing antigen, and bind to the antigen at sites usually referred to as epitopes or determinants. The number of epitopes varies from antigen to antigen, however aside from the limitation imposed on antigens having partial homology with self proteins, the majority of the surface is now thought to be potentially antigenic (Berzofsky, 1985). Epitopes are classified into two categories: (1) sequential or continuous epitopes which consist of a linear sequence of amino acids homologous with the inducing antigen, and (2) assembled epitopes, where the site of antibody binding consists of amino acids distant in the linear sequence but brought together by folding (tertiary structure).

Identification of sequential epitopes is readily accomplished using a complete set of all possible overlapping synthetic peptides of a given length homologous with the sequence of the antigen of interest. With the proviso that the length of the peptides is equal to or greater than the longest

sequential epitope, ELISA testing of this set of peptides with a given serum specific for that antigen will identify the antibody-binding peptides defined by that serum. The relationship between the antibody-binding peptides and sequential epitopes of the antigen is then established by methods such as competitive inhibition of binding using the whole antigen, use of monospecific antibody preparations, and by demonstrating with sufficient appropriately chosen negative controls that the antibodies binding to the peptides are antigen-specific.

The size of sequential epitopes in terms of number of amino acids has been estimated from detailed studies of antibody-binding peptides and from analyses of the size range of the antigen combining site of those antibodies for which the structure has been solved. The present consensus is that sequential epitopes are from 5 to 8 residues long (Kabat, 1970; Schechter, 1971). It follows that octapeptides should be used if all sequential epitopes are to be detected, whereas better resolution can be gained from scans of shorter peptides.

Scanning a protein of n residues thus requires the synthesis and testing of $(n-7)$ octapeptides, whereas $(n-5)$ hexapeptides constitute a complete scan at the shorter peptide length. For example, Fig. 2*A* is a scan of the model antigen myohemerythrin (Geysen et al., 1987) at hexapeptide length, using hyperimmune rabbit serum. It can be seen that certain peptides bind these rabbit antibodies with a good signal to background ratio. It is also clear that the large majority of peptides is essentially unreactive with any of the anti-myohemerythrin antibodies in this serum, a finding which was confirmed by the absence of interactions when a prebleed serum was tested on the same peptides (data not shown). Fig. 2*B* shows a

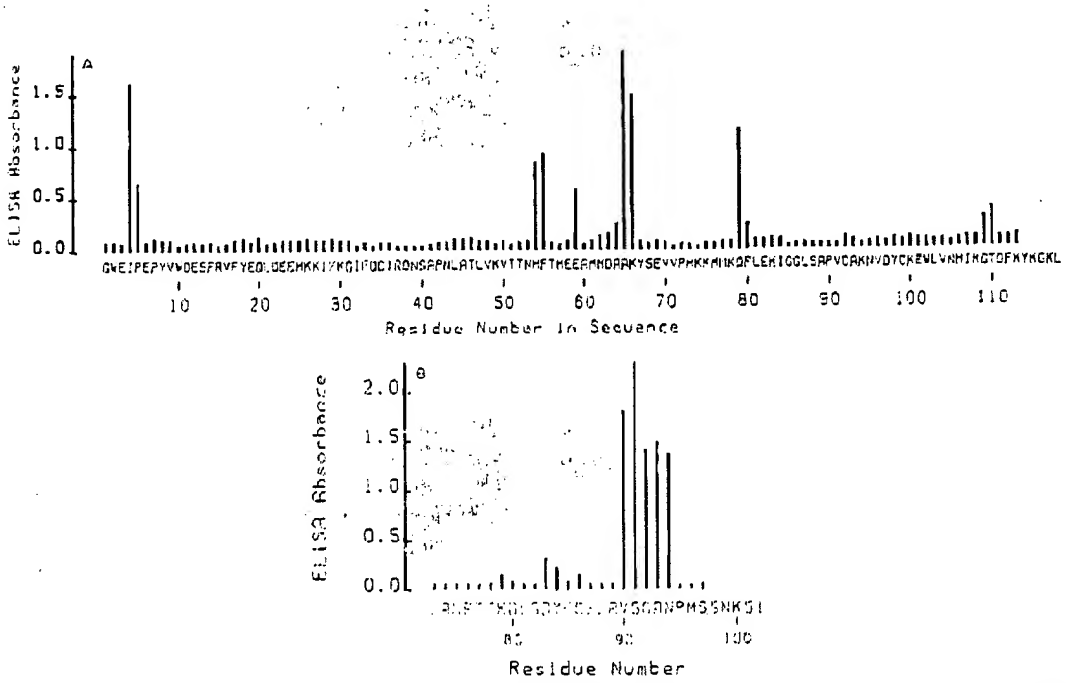

Fig. 2. Scans of hexapeptides homologous with the sequences of myohemerythrin (*A*) or Thy 1.1 (*B*). *A*: Peptides were reacted with a 1/1000 dilution of rabbit anti-myohemerythrin serum and the ELISA developed with OPD/hydrogen peroxide substrate (materials and methods section). B: Peptides were reacted with a 1/400 dilution of anti-peptide 88 serum (Alexander et al., 1983) and the ELISA was developed with ABTS/hydrogen peroxide substrate (materials and methods section). Each peptide is identified by both the number in the sequence and the single letter code of its amino-terminal residue. Thus, the sequence appearing on the ordinate is the sequence of the protein, commencing with the amino terminus at the left. The five carboxy terminal residues are shown for completeness.

scan, at the hexapeptide length, of a small region of the mouse T cell marker antigen Thy 1.1. The antiserum used in this example was an anti-peptide serum prepared against peptide ^{79}KDEGDYFCELRVSGANPMS^{97} homologous with this region of the protein (Alexander et al., 1983). Strong binding of the anti-peptide antibodies is seen with the five hexapeptides (^{90}VSGANP^{95} to ^{94}NPMSSN^{99}) adjacent to but not including those hexapeptides containing R^{89}, the latter peptides being only weakly reactive. This finding where the antipeptide serum only reacts with peptides common to the two Thy antigens, could explain the inability of this serum to distinguish between Thy 1.1 and Thy 1.2 in immunoblots (Alexander et al., 1983). The only amino acid difference between these antigens occurs at residue no. 89, being arginine in Thy 1.1 and glutamine in Thy 1.2.

Because of the large number of negative controls in any given scan, a 'test background' is quickly established, e.g., by ranking the absorbance values and averaging the lowest 25% of the values. Additional negative controls are readily tested, e.g., peptides from an unrelated antigen or 'nonsense' peptides, and our experience has been that the background from such additional negative controls agrees with the background established from the lowest 25% within a scan. We have not observed the non-specific binding associated with particular residues occurring near the amino terminus of solid-phase-bound peptides as reported by Shi et al. (1984).

We have not yet definitively established the significance of the absolute value of the absorbance on any given positive peptide. Under the ELISA conditions used (materials and methods section), we find that the net absorbance (after subtraction of background) is directly proportional to the concentration of the serum. The relative importance of antibody affinity for the peptide, or antibody concentration, in determining the absolute value is at present unknown. In a few examples studied (Geysen et al., 1986b; Rodda et al., 1986b), some monoclonal antibodies reacted with peptides in this system gave titers orders of magnitude higher, and some gave lower titres, than with native antigen. Clearly, the sensitivity of detection of epitopes is affected by the dilution chosen for the test of the antiserum, and a more useful representation of the results may be to titer the antibodies reactive with each peptide (Geysen et al., 1987).

The scanning approach presents a much larger database of information on peptide epitopes than was heretofore available. As a result, conclusions drawn from the limited information previously available will have to be modified, for example the 'established' epitopes of the widely studied antigen sperm whale myoglobin (Atassi, 1984) are not the major ones, nor are they conserved between responding species (Berzofsky et al., 1983; Rodda et al., 1986a).

Multiple length scanning (window / window + 1 analysis)

An alternative description of the scanning procedure is to imagine the protein to be stretched out in a line, and that we are looking at the sequence through a 'window' of a defined number of residues in width. By moving the window along the sequence in a step-wise fashion, the antibody-binding activity of each 'view' can be determined for the serum used in the test. Scanning multiple sets of peptides which differ only in peptide length is analogous to viewing the protein through multiple windows of differing width. The data collected from this procedure can be usefully analyzed to determine the differential activity of the view from two windows differing in width by one residue. From this the effect of the addition of the extra residue can be gauged in the context of those residues constant to each view. By aligning, at their amino terminal ends, peptides differing by one residue in length, the set of residues which affect binding of antibodies when they occur at the carboxy terminus of a peptide is identified. Aligning the peptides at their carboxy terminal ends then identifies the set of residues which have an effect when they occur at the amino terminus of the peptide. By this approach, the boundaries or ends of minimal antibody-binding sequences are readily determined. Residues, adjacent to the minimal antibody-binding sequence, which contribute significantly to the strength of binding are also revealed.

Fig. 3 shows an example of a multiple length scan of a small portion of the swMb sequence,

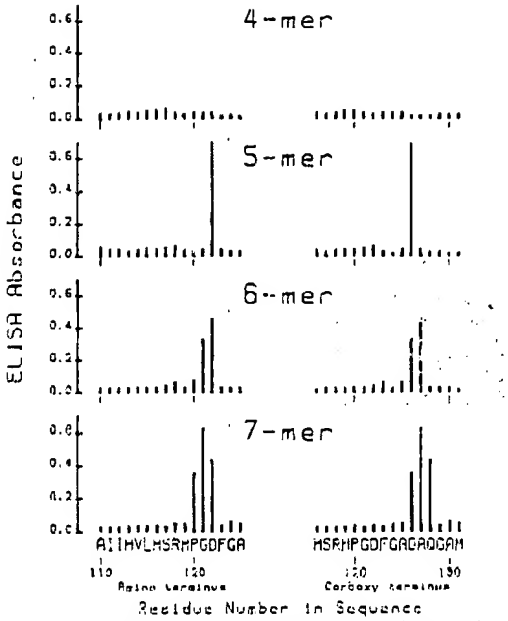

Fig. 3. Scans at multiple peptide lengths of peptides homologous with residues 110–131 of the swMb sequence. Peptides were reacted with a 1/800 dilution of rat anti-swMb serum (rat no. 118: Rodda et al., 1986a) and the ELISA developed with OPD/hydrogen peroxide substrate. Peptides in the left half of the figure are identified as in Fig. 2. Peptides in the right half of the figure are identified by the residue number and single letter amino acid code of their carboxy terminal residue, and are aligned at their carboxy termini. The lengths of the peptides are given above the scans, e.g., tetrapeptides are identified as 4-mer, pentapeptides as 5-mer, etc.

tested with a rat anti-swMb serum. It can be seen that there are no tetrapeptides in this region which bind these rat anti-swMb antibodies, but one pentapeptide, ^{122}DFGAD^{126} does. Window/window + 1 analysis applied to the tetra- and pentapeptides, by aligning the peptides at their amino termini, identifies residue D^{122} as critical to the binding interaction. Similarly, aligning the peptides at their carboxy termini identifies as a critical residue D^{126}. Furthermore, window/window + 1 analysis applied to the penta- and hexapeptide sets demonstrates that residues G^{121} and A^{127} are not directly involved in antibody binding. Similarly, analysis of the hexa- and heptapeptide sets demonstrates that residues P^{120} and Q^{128} do not contribute. Taken altogether these results suggest the presence of a single sequential epitope, comprising the five residues ^{122}DFGAD^{126}.

This type of analysis is most useful when epitopes are clearly separated by non-reactive sequences, otherwise, as in the case of overlapping epitopes, the contribution of a residue to binding cannot easily be ascribed to a particular epitope.

Replacement set analysis

Whilst the multiple length scanning technique provides basic information about the location and the boundaries of epitopes, no information about the contribution to antibody binding of the other individual residues within the epitope is obtained. This information is readily determined, for any residue in a reactive peptide, by comparing the antibody-binding ability of peptides which are synthesized with alternative residues in that location. If antibody binding is lost when the original (parent) residue is replaced with residues of dissimilar character, our interpretation is that this residue contributes directly to the interaction, i.e., it is a 'contact residue'. The converse, where antibody binding is essentially independent of the particular residue present in that location, is interpreted to indicate that the side chain of this residue is not in direct contact with the antigen-combining site of the antibody. An exception to the above categories of contact residues and non-contact residues occurs occasionally when the parent residue is one of the small amino acids glycine, serine or alanine. Replaceability is often limited to this trio. We have interpreted this to indicate that the conformation of the peptide, necessary for binding to the antibody, cannot be adopted when that residue has a bulky side chain.

The replacement set analysis thus involves systematic synthesis of a comprehensive set of peptide analogs, each analog differing from the parent peptide at only one location. This is done for each location (residue) of the parent peptide. As the 20 genetically coded amino acids represent residues of diverse properties, when compared for size, shape, hydrophilicity/hydrophobicity etc., they are sufficiently diverse replacements for the parent residue and allow a systematic evaluation of analogs. For a replacement set based on a parent sequence six residues long we therefore synthesise 120 peptides, including six copies of the parent sequence incorporated automatically as positive

controls, by using all 20 amino acids at each location.

Fig. 4 shows results of an ELISA with a replacement set consisting of the 100 peptide analogs of the antibody-binding swMb pentapeptide shown in Fig. 3 (DFGAD). The replacement set was reacted with rat anti-swMb serum taken from the same animal 5 days before the serum used in generating Fig. 3. It is clear that the only analog as effective at binding anti-swMb antibodies as the parent pentapeptide is the peptide DFGSD, with S in place of the A, a relatively conservative replacement. In this example, the A is the most replaceable residue, although some of the analogs in which the amino terminal D or the F are replaced also bind a significant amount of antibody. The G is an example of a completely non-replaceable residue. It is frequently possible to discern relationships between the residues partly or wholly acceptable as replacements, for example, glutamic acid as a replacement for the carboxy terminal aspartic acid. A more detailed treatment of the analysis of replacement set results is in preparation.

There is clear agreement between the replacement set analysis and the window/window + 1 analysis (above) of this peptide. Both approaches identify the terminal aspartic acid residues as vital to the epitope. However, the replacement set analysis clearly provides much more insight into the fine structure of the epitope.

A difficulty inherent in the analysis of a replacement set using polyclonal antibodies is the possibility of the presence of populations of antibodies which will accept different patterns of replacements. At this fine level of discrimination, one peptide is effectively a single epitope for different paratopes (antibody combining sites). Monoclonal antibody preparations, having only one paratope, do not suffer from this complication. Fig. 4 shows the ELISA test results with the 120 hexapeptides of the replacement set of peptide QGGLED, a peptide recognized by an MAb raised against the repeat sequence of a

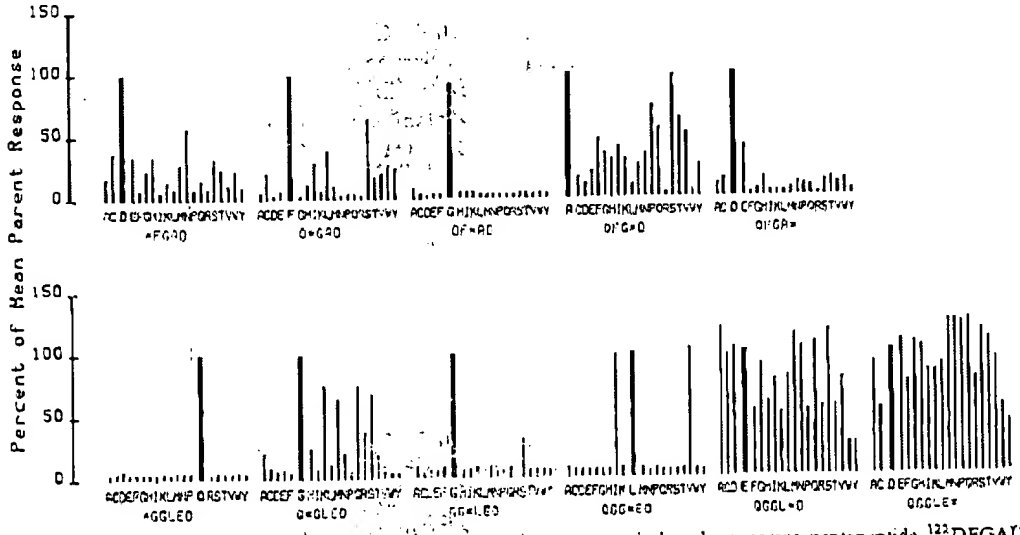

Fig. 4. Replacement set analysis of two epitopes. The upper replacement set is based on parent pentapeptide ^{122}DFGAD^{126} homologous with swMb. Each block of 20 ELISA values represents the results with peptides containing the single amino acid substitution identified by the single letter code beneath each bar. The position of the substitution is indicated by an asterisk in the sequence given under that block. The homologous amino acid is emphasised. ELISA values for the five copies of the parent sequence have been averaged and taken as 100% for the purposes of comparison with replacement analogs. The peptides were tested with 1/800 dilution of serum from rat no. 118 (Fig. 3) and the ELISA developed with OPD/hydrogen peroxide substrate. The lower replacement set is based on parent hexapeptide QGGLED, a portion of a repeat sequence (Bushell et al., 1987) of *Plasmodium falciparum* S-antigen. These 120 hexapeptides were tested with a 1/800 dilution of a monoclonal ascites fluid and the ELISA developed with OPD/hydrogen peroxide.

malarial S-antigen (Bushell et al., 1987). In this case, the contact residues are Q, the second G, and L. The patterns of replaceability are very clear: no residue will substitute for the Q, only the small hydrophilic S will substitute for the second G, and the aliphatic hydrophobic I and V are equally acceptable in place of the L. Peptides with alternative residues to either the E or D all retain some ability to bind this MAb.

Mimotope strategy

Modern techniques allow the rapid deduction of amino acid sequences by the direct translation of the genetic code corresponding to a final protein product. Each of the preceding strategies for the identification and analysis of antibody-binding peptides depends on the availability of the primary sequence for the protein antigen under study. However, extensive scanning of diverse MAbs has shown that the majority (approaching 90%) fail to react (Geysen et al., 1986a; unpublished work). We have interpreted these findings to indicate that the majority of antibodies recognize assembled epitopes, and by extrapolation, that a similar proportion of antibodies comprising a polyclonal response also recognizes assembled epitopes. In order to overcome the need for sequence information, a limitation of the methods described above, the mimotope strategy was formulated (Geysen, 1985; Geysen et al., 1986b). It is likely that the only essential criterion for effective binding of antibody to a peptide is that complementarity between the antigen-combining site of the antibody and the molecular surface of the binding peptide is maintained in regard to both shape and charge. It follows that antibody binding peptides deduced without recourse to sequence information should be defined as 'mimotopes', or mimics of the epitope which induced the antibody, rather than assuming that they are an accurate reproduction of that epitope.

In our experience, it is not necessary to scan peptides any longer than octapeptides to demonstrate all continuous epitopes. This suggests a direct way to identify antibody-binding peptides, namely, to start with the total repertoire of octapeptides which can be made using the 20 genetically coded amino acids, i.e., 20^8 peptides. This strategy could be expected to give results with antibodies to both sequential and assembled epitopes. Even were it possible to synthesize this very large number of peptides, the testing would require a prohibitive quantity of antibody. The problem of the large number of peptides involved in the initial screen was overcome by synthesizing a multiplicity of peptides onto a single rod (Geysen et al., 1986a; Geysen et al., 1986b). Successive syntheses led to the progressive identification and optimization of the sequence of an antibody-binding peptide from the many combinations present on the original rod. However, the first application

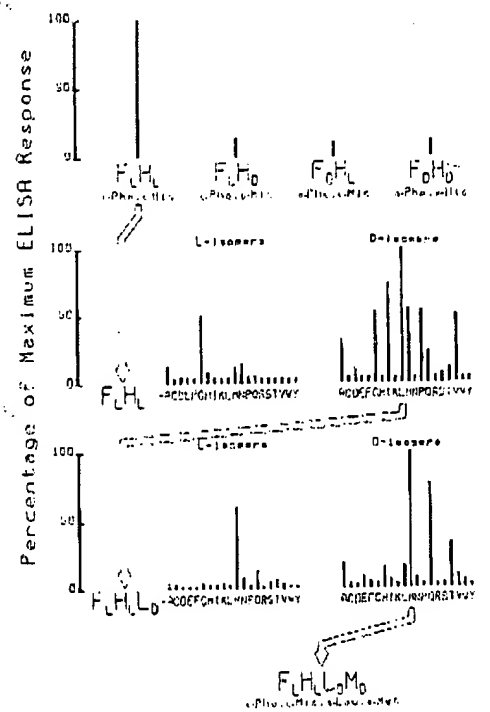

Fig. 5. A priori development of a mimotope. Top: ELISA results with all four stereoisomeric pairings of dipeptide FH, using anti-rabies virus monoclonal ascites fluid 101-1 (Rodda et al., 1986b) at a dilution of 1/800. Middle: ELISA results with tripeptides comprising sequence (L-Phe)-(L-His)-X, where X is one of the 20 genetically coded amino acids, in either L- or D-stereoisomeric form. MAb 101-1 tested at a dilution of 1/7000. Bottom: ELISA results with tetrapeptides comprising sequence (L-Phe)-(L-His)-(D-Leu)-X where X is as above. MAb 101-1 tested at a dilution of 1/1000000. All tests were developed with OPD/hydrogen peroxide, and the optical densities expressed as a percentage of the maximum for that set of peptides.

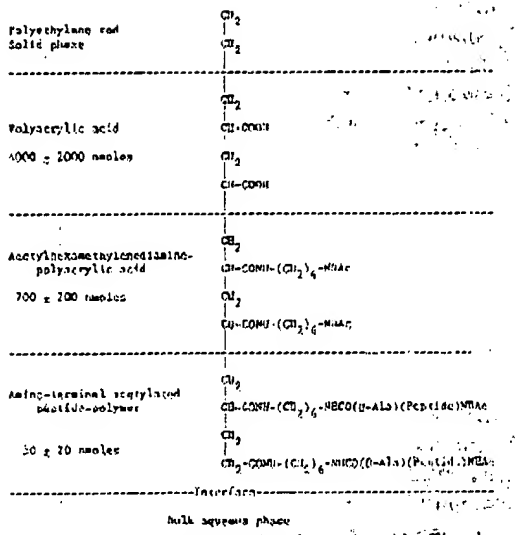

Fig. 6. Composition of the rod/polymer/peptide. The chemical structural formulas of the zones around tip of the polyethylene rod are shown, along with the quantity (mean and range) of each. Ac = acetyl group; β-Ala = β-alanine.

of the method quickly demonstrated the limitations in mimicking assembled epitopes using peptides whose residues were restricted to the L-optical isomers of the genetically coded amino acids (Geysen et al., 1986b).

With the demonstration that antibodies bind to dipeptides in a reproducible manner and with a high degree of specificity (Geysen, 1985), it seemed likely that a strongly binding peptide could be built up from the optimum binding dipeptide unit. Having established that optical isomerism of the amino acids used has a powerful influence on the strength and specificity of antibody binding by dipeptides, it was logical to include in the starting repertoire both the L- and D-optical isomers of the genetically coded amino acids.

In order to extend a selected, strongly binding dipeptide to give a mimotope of higher specificity and affinity, sets of longer peptides based on that dipeptide were synthesised. These 'extension sets' consisted of approximately 184 peptides, comprising both the amino terminal and carboxy terminal addition to the dipeptide of members of a set of diverse amino acids, with or without the insertion of β-alanine as a spacer. The set of amino acids comprised the L- and D-isomers of each genetically coded amino acid, and a small selection of amino acids which do not occur in proteins. Fig. 5 illustrates the development of a tetrapeptide mimotope for a MAb directed against rabies virus (Rodda et al., 1986b), including two cycles of extension set synthesis. Only the 'direct' extension results at the carboxy terminal end are shown, i.e., those in which there was no spacer, since neither the spaced carboxy-terminal extensions nor the amino terminal extensions led to increases in binding comparable to those shown. As the tetrapeptide mimotope obtained contains two D-amino acids, it could not have been arrived at by testing peptides homologous with the rabies virus antigen, nor would it have been feasible to predict the mimotope from the tertiary structure of the antigen, were it known. The mimotope is therefore unique in its structure and method of derivation.

Discussion

The rod system described is clearly a combination of the well established procedures of solid-phase peptide synthesis as first demonstrated by Merrifield (1963) and the ELISA developed by Engvall and Perlmann (1971). That some antibodies would bind to peptides while these were still attached to the resin used for their synthesis was originally demonstrated by Smith et al. (1977). The observation that the length and physicochemical properties of linking molecules are important parameters affecting the affinity purification of proteins when using carrier-bound ligands, was also considered in formulating the synthesis and testing procedure.

The kinetics of the amino acid coupling reactions in dimethylformamide solution indicate that these are diffusion controlled (unpublished observations), consistent with the concept of reactions occurring in a deep polymer matrix solvated by DMF. The amine groups available for synthesis are deliberately limited by using a relatively short coupling time for the BOC-β-alanine linker. Acetylation blocks any further reaction of amine groups deep in the polymer, leaving a surface layer of BOC-protected amine. After synthesis, when the peptide-polymer is solvated by water, peptides are present in a surface zone with solvated

polymer on one side and the bulk aqueous phase on the other (Fig. 6). This environment should favor the conformational freedom of these synthesized peptides, and should not present any steric restrictions on the access of the reacting protein (e.g., antibody) to peptide.

It is our experience that using the methods described, interactions between peptides and antibodies are more reliably detected than when the peptide is first adsorbed onto a plastic surface in preparation for a more conventional ELISA. We suggest that it is unlikely that a very small peptide (say smaller than eight residues) could simultaneously bind both to an antibody and non-covalently to a plastic surface. The practice of first coupling a peptide to a protein carrier and then using this conjugate to coat the plate in preparation for an ELISA is approximately equivalent to the peptide-polymer combination used here. In a recently completed study of antigen-antibody interaction, good agreement between the various methods used for the detection of peptide-antibody interactions was obtained (Getzoff et al., 1987; Geysen et al., 1987). Experiments in which the length of the linking molecule was varied, and in which linking molecules with varying degrees of hydrophilic and/or hydrophobic character were tried, showed that the test results could be reproduced on alternative linking molecules (unpublished results).

The possibility that synthetic peptides may be used as vaccines is attractive, and has focussed interest on gaining a better understanding of the molecular basis for the immunogenicity of proteins. In particular, attention has been directed at identifying relationships between measurable properties of a protein and its immunogenic sites (Berzofsky, 1985). Of the several relationships suggested, the majority require that the X-ray crystallographic structure of the protein is known, which severely limits their usefulness. With the ready availability of protein sequences, the most widely used algorithm is based on the purported likelihood of finding a sequential epitope at the site of a local maximum in the hydrophilicity profile (Hopp and Woods, 1981). More recently, using a more complete and systematically obtained database of the sequential epitopes for the model protein antigen myohemerythrin, we have shown that no statistically significant relationship based on hydrophilicity could be demonstrated (Geysen et al., 1987).

We now suggest that starting with the known amino acid sequence of a protein of interest, it is eminently more satisfactory to locate the sites corresponding to the sequential epitopes experimentally. In 10 working days we are able to complete the synthesis of the total set of overlapping octapeptides corresponding to any protein of known sequence. Then, depending on the number of copies of this set synthesized, one or more sera can be tested each day. In a relatively short time, this procedure generates a comprehensive picture of the location of sequential epitopes, and at the same time provides information as to the frequency with which each is recognized by individual members of a species.

In our laboratory, the identification of sequential epitopes is usually followed by the synthesis of a replacement set to determine the contact residues, i.e., those amino acids which are critical to the interaction of each peptide with the reacting antibody population. Where peptides corresponding to an epitope are to be evaluated for immunogenicity, the knowledge of precisely which residues are essential for the interaction with antibody becomes important and could limit the methods used to conjugate the peptide to a carrier molecule. For example, when the epitope is shown to contain a lysine residue which is essential to the interaction with antibody, a conjugation method which would link through the lysine side chain should be avoided.

A further use of the replacement set of analogs of a peptide epitope is that it allows a ready comparison of the specificity for individual residues between antibodies raised against the intact, native protein and those raised against the peptide immunogen (Geysen et al., 1985).

The recent X-ray crystallographic solution of several antigen-antibody complexes has raised some questions about the validity of the concept of sequential epitopes (Amit et al., 1986; Colman et al., 1987). The large surface area of antigen in contact with antibody, and the large number of residues implicated for the lysozyme-Fab complex, 16 and 17 residues for lysozyme and Fab respectively, do not at first glance appear consistent with

our observation, using the scanning technique, that octapeptides are the longest peptides needed to detect all continuous epitopes. Furthermore, using the replacement set procedure, we have not observed any epitopes in which more than five residues were contact residues.

Amit et al. (1986) reported that binding was lost between the Fab and California quail lysozyme which differs from chicken lysozyme in only a single residue (Q^{121} changed to H) among those that are in contact with the antibody. This observation was contrary to their expectations because the space in the complex occupied by the glutamine was estimated to be able to accomodate the histidine. Furthermore, this histidine should have been able to form a hydrogen bond as did the glutamine in hen egg-white lysozyme. This alone suggests that a large majority of the binding energy is derived from the deep penetration of the hydrophobic cavity by the glutamine, and by the formation of the hydrogen bond in the floor of the cavity. A further observation was that, of the total of 16 residues of lysozyme which formed the interface in the complex, only four residues involved in hydrogen bonding contact were sufficiently close to antibody residues (≤ 2.5 Å) to contribute significantly to the overall binding energy.

Clearly, as a consequence of the way in which we identify them, the contact residues are those which contribute in a positive way to binding with antibody. We propose that the contact residues constitute the complete epitope. Residues which do not contribute significantly to the overall binding energy will appear generally replaceable when tested in the context of a conformationally free peptide. In a native protein antigen, with its conformational restraints, this replaceability may not apply. In this case the final binding conformation of the contact residues may be dependent on the properties of those residues adjacent in sequence or space. These near-neighbour residues may preclude the necessary molecular movements in the antigen from taking place, or sterically prevent the close approach of the antibody. In this way it may appear (e.g., from comparison of sequence variants) that near-neighbour but non-contact residues make a positive contribution to complex formation, when their effect is indirect. For example, a 'critical' near-neighbour residue may simply permit a conformational alteration of the antigen required for binding to that particular antibody.

The mimotope procedure constitutes a radical departure from established methods. It does not require any information about the structure of the antigen (or ligand in its broader application), merely a source of the antibody or receptor. Delineation of the mimotope proceeds in an iterative fashion with the possibility at each stage of making choices which influence the nature of the final molecule(s). If, instead of proceeding from the optimum binding peptide at each stage, a near optimum peptide which comprises residues with specific characteristics is chosen, these characteristics are maximized in the final mimotope. For example, in order to enhance resistance to proteolytic degradation, it may be advantageous to 'drive' the final mimotope to include the maximum number of D-optical isomers of amino acids while still maintaining an adequate affinity for the antibody. Alternatively the inclusion of several non-genetically coded amino acids may be advantageous.

The relationship between the mimotope and the epitope which gave rise to that antibody is not yet clear. In a study with the Mcg Bence-Jones light-chain dimer, Edmundon et al. (1984, 1985) clearly demonstrated that this immunoglobulin-like cavity was able to bind a diverse range of ligands. These included a range of multi-ringed aromatic dyes as well as several peptides of varied amino acid composition. We have now determined mimotopes for the Mcg light-chain dimer and the solution of their structures, as bound in crystals of the dimer, is underway. These results will reveal the precise location of the bound mimotopes in an immunoglobulin-like binding cavity. However, the precise relationship between a mimotope and the actual epitope is likely to remain unknown except for those cases where mimotopes are determined for antibodies for which the epitope has been defined from the solution of the X-ray crystallographic structure of its complex with the antigen.

We have described a method of peptide synthesis which can be carried out by people with a limited knowledge of chemistry. The number of peptides which can be synthesized concurrently and in a format which is directly testable by ELISA, is significantly greater than by alternative

methods. The synthesis options outlined have been used to provide a greater understanding of the basis for immunogenicity and the nature of antigen-antibody binding. The mimotope strategy has yet to identify peptides useful as synthetic vaccines, however at this early stage we suggest that its greatest impact may be in the field of defining small peptide mimics for a variety of peptide hormones.

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Identification of Antigenic Epitopes in a Surface Protein Antigen of *Streptococcus mutans* in Humans

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The reactivities of antibodies in human serum and saliva to a cell surface protein antigen (PAc) of *Streptococcus mutans* and synthetic peptides covering the PAc molecule were examined. Both an enzyme-linked immunosorbent assay (ELISA) and Western blotting (immunoblotting) showed that all the serum samples from five adult subjects harboring serotype c *S. mutans* in their oral cavity reacted with recombinant PAc (rPAc). On the other hand, the serum from a 4-month-old infant did not react with rPAc in ELISA. The immunoglobulin A (IgA) antibodies in saliva samples from the five adult subjects reacted with rPAc. However, in saliva samples from these subjects, the titers of IgA antibody to rPAc did not correlate with the titers of serum antibody to the antigen. To map continuous antigenic epitopes in the PAc molecule, we synthesized 153 decapeptides covering the entire mature PAc molecule, 121 overlapping decapeptides covering the alanine-rich repeating region (A-region) of the PAc molecule, and 21 overlapping decapeptides covering the middle region (residues 824 to 853) according to multiple pin-coupled peptide synthesis technology. Of 153 decapeptides covering the mature PAc, 27 decapeptides showed a strong reaction with the antibodies in serum from the adult subjects. The epitope-scanning patterns in the serum samples from these subjects were also very similar to each other. The antigenic epitope patterns in the saliva resembled those in the serum. However, the ELISA titers of salivary IgA antibodies to these decapeptides differed from the titers of the serum antibody. Of the 121 overlapping decapeptides covering the A-region, 27 decapeptides showed a positive reaction with the antibodies in serum from the adult subjects. All of these 27 decapeptides had either one or two of the five common sequences YQAXL, NADAKA, VQKAN, NNAKNA, and IKKRNA. Six decapeptides of the 21 overlapping decapeptides covering the middle region reacted strongly with the serum antibodies from a high PAc responder, and each of the six decapeptides had one of the two common sequences KVTKEKP and VKPTAPTK. These epitopes might therefore be relevant to the humoral responses against the PAc protein during natural infection with *S. mutans* in humans.

Streptococcus mutans has been strongly implicated as one of the causative agents of dental caries and is frequently isolated from human dental plaque (16, 29). *S. mutans* possesses various cell surface antigenic substances. Among these antigens, a 190-kDa cell surface protein antigen that has been variously designated antigen I/II (42), B (45), IF (20), P1 (12), SR (1), PAc (38, 39), and MSL-1 (10) was shown to be an effective dental caries vaccine in monkeys (28, 46). The application of mouse monoclonal antibodies against the antigen to tooth surfaces was demonstrated to inhibit either the subsequent implantation of *S. mutans* or the recolonization by indigenous mutans streptococci in monkeys and humans (27, 30). The surface protein antigen is considered to participate in attachment of the streptococcal cell to acquired pellicles on tooth surfaces (22, 26). The surface protein of *S. mutans* shows a serological cross-reactivity with a 210-kDa surface protein of *Streptococcus sobrinus* named SpaA (18) and PAg (37, 49, 51).

The complete nucleotide sequence of the gene for the 190-kDa protein antigen of *S. mutans* serotype c has already been determined by two groups (21, 39). The *pac* gene consists of 4,695 bp and codes for a 170,773-Da protein (39). The gene product contains a putative signal peptide consisting of 38 amino acids, resulting in a 166,817-Da mature protein. Two internal repeating amino acid sequences are present in the PAc: one repeating region (the A-region), located in the N-terminal region (Thr-219 to Lys-464), is rich in alanine, while the other (the P-region), located in the central region (Thr-851 to Glu-967), is rich in proline.

The nucleotide sequencing of the *pac* gene of *S. mutans* has led to the identification of functional domains and antigenic epitopes in the PAc molecule (9, 33–35, 40, 47). We previously showed that the intranasal immunization of BALB/c mice either with a synthetic peptide corresponding to residues 301 to 319 of the PAc protein, coupled to a cholera toxin B subunit, or with recombinant PAc (rPAc) and a free cholera toxin B subunit suppresses the colonization of murine tooth surfaces by *S. mutans* (48). Moreover, epitope scanning with anti-rPAc sera from various strains of mice and many decapeptides covering the mature PAc molecule suggested that the murine immune responses to the peptides might be either restricted or dominated by the major histocompatibility complex class II gene haplotypes (40, 47). Therefore, in order to apply the PAc as an anti-*S. mutans* vaccine to human beings, the immune responses to this protein in humans need to be elucidated. However, little is still known about the immune responses in humans to the PAc protein of *S. mutans*.

In this study, we examined the reactivities of antibodies in samples of serum and saliva from adult subjects harboring *S. mutans* in their oral cavity with the PAc by both an enzyme-

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TABLE 1. Amino acid sequences of 20-mer synthetic peptides containing antigenic epitopes of PAc

Peptide	Position[a]	Amino acid sequence[b]
PAc(96–114)	96–114	LDQAAKDAKSAGVNVVQDAC
PAc(331–349)	331–349	KATYEAAVAANNAKNAALTC
PAc(513–531)	513–531	EPNANLSLTTDGKFLKASAC
PAc(623–641)	623–641	SKIVYKYTVDPKSKFQQGQKC
PAc(764–782)	764–782	PNSWYGAGAIKMSGPNNHVC
PAc(824–842)	824–842	AVNVPKVTKEIQPTPPVKPTC
PAc(855–853)	855–855	PTPPVKPTAPTKPTYETEKC
NA(464–482)[c]		WFGKHRGQGGSNPKFENIA

[a] Position according to Okahashi et al. (39).

[b] The sequences are given in one-letter code. A cysteine was placed at the C terminus of each of these except the last.

[c] An irrelevant peptide derived from the amino acid sequence of Epstein-Barr virus nuclear antigen (2).

linked immunosorbent assay (ELISA) and Western blotting (immunoblotting). Furthermore, we synthesized 153 decapeptides covering the entire mature PAc molecule, 121 overlapping decapeptides covering the A-region of the PAc, and 21 overlapping decapeptides covering the middle region, and determined the reactivities of the serum and salivary antibodies from the subjects with these decapeptides to identify the human continuous B-cell epitopes in the PAc protein.

MATERIALS AND METHODS

Human serum and saliva. Sera were obtained from 11 healthy donors between 25 and 43 years old and from a 4-month-old infant. Whole saliva was collected from five adult donors by paraffin stimulation in a chilled container and was clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. The human leukocyte antigen types of subjects A to E, respectively, were as follows: A11, Aw33, B44, DRw6(w13), DRw8, DRw52, DQw1; A11, Aw33, B44, Cw7, DR2(w15), DRw6(w13), DRw52, DQw1(DQw5); A24, A11, Bw52, B7, Cw7, DR1, DR2(w15), DQw1(DQw5, DQw6); A2, A31, Bw48, Cw4, DR2(w15), DR9, DRw53, DQw1(DQw6); and A24, A31, Bw61, Bw59, Cw1, Cw3, DR1, DR9, DRw53, DQw1(DQw5), DQw9. The average number (mean ± standard deviation) of decayed, missing, or filled teeth in the 11 adult subjects was 11.0 ± 4.8. The numbers of decayed, missing, or filled teeth in subjects A to E were 3, 10, 9, 19, and 12, respectively.

Isolation of mutans streptococci. Swab samples, collected with a cotton applicator from the teeth or oral cavity, were serially diluted with 0.15 M NaCl and plated on a sucrose-bacitracin-supplemented mitis-salivarius (MS-SB) agar (15). The MS-SB plates were incubated at 37°C for 48 h. The colonies from each individual were purified by being streaked twice on the MS-SB agar and cultured in 5 ml of brain heart infusion (Difco Laboratories, Detroit, Mich.) broth at 37°C for 16 h. The identification of mutans streptococci and the serotyping of these isolates were performed as described by Hardie (17) and Masuda et al. (31), respectively.

Antigens. *S. mutans* MT8148 (serotype c) was grown at 37°C for 18 h in a diffusate medium of brain heart infusion broth. The cells were harvested by centrifugation, washed three times with distilled water, and lyophilized. rPAc was purified from the culture supernatants of transformant *S. mutans* TK18 by ammonium sulfate precipitation and chromatography on DEAE-cellulose (22). Seven 20-mer peptides corresponding to parts of the amino acid sequence of the PAc (39) and an irrelevant 19-mer peptide, NA(464–482), derived from the amino acid sequence of Epstein-Barr virus nuclear antigen (2) were synthesized by the solid-phase procedure (32) and purified by high-performance liquid chromatography (Table 1). A cysteine was placed at the carboxyl terminus of all 20-mer synthetic peptides. The purity of the peptides was assessed by high-performance liquid chromatography and amino acid analysis.

ELISA. For the ELISA, 96-well microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 100 μl of whole cells of *S. mutans* MT8148 (100 μg/ml), rPAc (10 μg/ml), or peptide (1 μg/ml) in 50 mM carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed with phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 (PBST) and blocked with PBST containing 5% (wt/vol) skim milk. After the plates were washed three times with PBST, twofold serial dilutions of human serum or saliva were added (100 μl per well) and the wells were incubated for 1 h at 37°C. The bound antibodies were detected with either alkaline phosphatase-conjugated goat anti-human immunoglobulin A [IgA(α)] (Organon Teknica, Malvern, Pa.), anti-human IgM(μ) (Zymed Laboratories, South San Francisco, Calif.), anti-human IgG(γ) (Zymed), or anti-human IgG (heavy and light chains) (Zymed) serum followed by the addition of a *p*-nitrophenylphosphate substrate solution (1 mg/ml). After 1 h of incubation at 37°C with the substrate, the A_{405} was measured with a microplate reader (MPR A4; Tosoh, Tokyo, Japan). The ELISA antibody titer was expressed as the $\log_2$ of the reciprocal of the highest dilution giving an A_{405} of 0.1 above the conjugate control (no sample added) after 1 h of incubation with the substrate (48). To determine the amount of Igs specific for whole cells of *S. mutans* or rPAc, the calibration curves for IgA, IgM, and IgG were made. In brief, 96-well microtiter plates were coated with 1 μg of either goat anti-human IgA(α) (Organon), anti-human IgM(μ) (Organon), or anti-human IgG(γ) (Zymed) serum. After blocking with 5% (wt/vol) skim milk, twofold serial dilutions of 1 μg of affinity-purified human IgA, IgM, or IgG (Chemicon International Inc., Temecula, Calif.) per ml were added. The bound Igs were detected as described above. The calibration curves were obtained by using the equation of log-logit transformation, and the interpolations of the data for the serum and saliva samples were fitted to the linear portion of the curve with a correlation coefficient of higher than 96% (41). In the inhibition assay of the binding of human serum antibodies to rPAc, the ELISA plates were coated with rPAc (1 μg per well). A 1/100 dilution of the serum from subject A was allowed to react with various concentrations of synthetic peptides or rPAc overnight at room temperature. The reaction mixtures were added to rPAc-coated wells (100 μl/ml), and then the plates were incubated for 1 h at 37°C. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) serum, followed by the addition of the substrate. The percent inhibition of ELISA was calculated by the following formula: 100 × [(A_{405} of human serum) − (A_{405} of human serum plus peptide or rPAc)]/(A_{405} of human serum).

Western blotting. Concentrated culture supernatants and cell extracts of *S. mutans* MT8148 were prepared as described by Ohta et al. (36). *S. mutans* MT8148 was grown at 37°C for 18 h in a diffusate medium of brain heart infusion broth. The cell-free culture supernatant was collected by centrifugation at 10,000 × *g* for 20 min at 4°C. Extracellular proteins were precipitated from the culture supernatant by addition of solid ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation, dissolved in distilled water, dialyzed against distilled water, and lyophilized. The dried culture supernatant, rPAc, and whole cells (50 mg [dry weight] per ml)

were suspended separately in a solution containing 8 M urea, 10 mM Tris hydrochloride (pH 6.8), 1% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) 2-mercaptoethanol and heated at 100°C for 3 min. The cell extract was then clarified by centrifugation. SDS-polyacrylamide slab gels were prepared as described by Laemmli (24). The culture supernatant (10 μg of protein), rPAc (2 μg of protein), and the cell extract (2.5 μl) were electrophoresed at 30 mA per gel for 50 min with a 7.5% (wt/vol) resolving and a 3% (wt/vol) stacking gel (90 by 80 by 1 mm) containing 0.1% (wt/vol) SDS and transferred to a nitrocellulose sheet by the Western blotting technique (5). Myosin (212 kDa), α-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa) were used as molecular mass markers. Nonspecific binding to the blots was blocked by incubation for 1 h at 20°C in TBS (20 mM Tris hydrochloride [pH 7.5], 500 mM NaCl) with 3% (wt/vol) gelatin. The blots were washed twice with TBST (TBS with 0.05% [vol/vol] Tween 20) and then incubated with human sera (1:20 in TBS–1% [wt/vol] gelatin) at 4°C overnight. After the blots were washed twice with TBST, they were incubated with goat anti-human IgG (heavy and light chains) conjugated to horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands). The blots were washed twice with TBST and once with TBS, and then substrate (3,3'-diaminobenzidine-hydrogen peroxide) was added.

Epitope scanning. A set of 153 decapeptides serially covering the complete amino acid sequence of mature PAc(39), a set of 121 overlapping decapeptides covering the A-region of the PAc molecule (residues 219 to 468), and a set of 21 overlapping decapeptides covering the middle region of the PAc molecule (residues 824 to 853) were synthesized with an epitope scanning kit (Chiron Mimotopes, Clayton, Australia) as reported by Geysen et al. (13). The reactivities of peptides coupled to multiple pins with human saliva (1:10 dilution) and sera (1:800 or 1:1,600 dilution) were determined by ELISA according to the instructions of the manufacturer. The bound antibodies were detected with either alkaline phosphatase-conjugated goat anti-human IgA(α) or anti-human IgG (heavy and light chains) serum. The data were expressed as the A_{405} after 1 h of incubation with the substrate.

RESULTS

Human antibody responses to *S. mutans* antigens. Antibodies to whole cells of *S. mutans* and rPAc in human serum samples were quantified by ELISA with class-specific Igs as references. The mean concentrations of IgG, IgA, and IgM antibodies to whole cells of *S. mutans* in the serum samples from 11 adults were 91.0 μg/ml (ranging from 13.4 to 372.6 μg/ml), 25.4 μg/ml (ranging from 3.5 to 113.0 μg/ml) and 10.2 μg/ml (ranging from 1.8 to 25.0 μg/ml), respectively. The concentrations of serum IgG, IgA, and IgM antibodies to whole cells of *S. mutans* in a 4-month-old infant were 0.07, 0.02, and 0.02 μg/ml, respectively. The mean concentrations of serum IgG, IgA, and IgM antibodies to rPAc were 16.6 μg/ml (ranging from 1.0 to 57.3 μg/ml), 6.3 μg/ml (ranging from 0.6 to 24.6 μg/ml) and 1.1 μg/ml (ranging from 0.2 to 4.1 μg/ml), respectively. The concentrations of the serum IgG, IgA, and IgM antibodies to rPAc in the infant were 0.04, 0.02, and 0.01 μg/ml, respectively. Total Ig concentrations in the sera from 11 adults averaged 14.8 ± 1.7 mg/ml (mean ± standard deviation), and the concentration in the infant's serum was 11.7 mg/ml. There was also a close correlation between the concentrations of serum class-specific antibodies to whole cells of *S. mutans* and those of antibodies to rPAc. The correlation

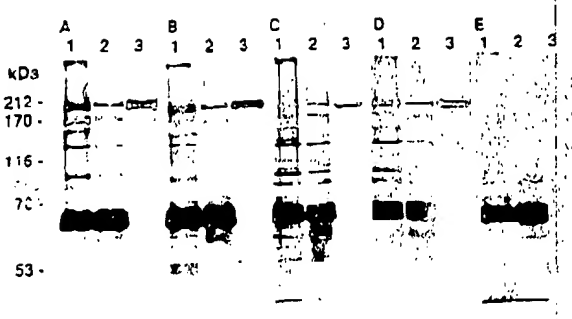

FIG. 1. Western blot analysis of both the cell extracts (lanes 1) and the culture supernatants (lanes 2) of *S. mutans* MT8148 and rPAc (lanes 3). The whole cells were suspended in 8 M urea–1% SDS–1% 2-mercaptoethanol and heated at 100°C for 3 min. The cell extracts were clarified by centrifugation. The culture supernatants were then concentrated by ammonium sulfate precipitation. rPAc was purified from the culture supernatants of transformant *S. mutans* TK18 by ammonium sulfate precipitation and chromatography on DEAE-cellulose. These antigens were electrophoresed on SDS-polyacrylamide slab gels and transferred onto a nitrocellulose sheet by an electrophoretic blotting procedure. The immobilized antigens were allowed to react with the sera (1:20) from five adult subjects (A to E). The antibodies which bound to the antigens were detected by solid-phase immunoassay with horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains).

coefficients of the IgG, IgA, and IgM antibody data were 0.961, 0.828, and 0.835, respectively.

On the basis of the titers of serum IgG antibody to rPAc, two high responders (subjects A and B), two medium responders (subjects C and D), and one low responder (subject E) were chosen from among the 11 adults. The reactivities of the sera from these subjects with cell extracts and culture supernatants of *S. mutans* and with rPAc were then analyzed by Western blotting with horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains) serum. The human serum antibodies reacted strongly with 190-, 70-, and 65-kDa antigens of *S. mutans* (Fig. 1). The reactivities of these sera with the 190-kDa PAc in Western blotting corresponded to those in ELISA (Fig. 2A). The titers of IgA antibody in saliva samples from these five subjects to rPAc did not correlate with the serum antibody responses to the protein antigen (Fig. 2B).

We examined whether these subjects were naturally infected with mutans streptococci. *S. mutans* was isolated from the samples of dental plaque from all the adult subjects, and all the *S. mutans* strains isolated in this study possessed the serotype c antigen described by Bratthall (4). Moreover, serotype g *S. sobrinus* as well as serotype c *S. mutans* was isolated from the dental plaque sample of subject D. Neither *S. mutans* nor *S. sobrinus* was recovered from the oral cavity of the 4-month-old infant.

Epitope scanning of the PAc molecule. To clarify the antigenic epitopes in the PAc molecule, we synthesized a set of 153 decapeptides covering the complete amino acid sequence of the mature PAc and used these decapeptides in the epitope-scanning analysis. Figure 3 shows the epitope-scanning patterns with serum samples from five adult donors infected naturally with serotype c *S. mutans* and from an infant. Serum (1:800 dilution) from subject A reacted with many decapeptides and showed positive reactions ($A_{405} \geq 1.0$) with the 27 decapeptides corresponding mostly to the N-terminal and the middle regions of the PAc (Fig. 3A). In particular, the serum gave positive reactions with five decapeptides corresponding to

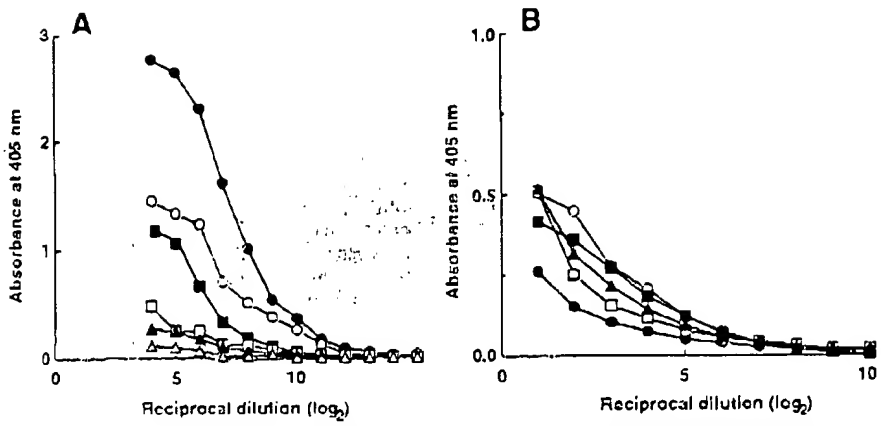

FIG. 2. Reactivities of rPAc with antibodies in serum (A) and saliva (B) samples from five adult subjects (●, subject A; ○, subject B; ■, subject C; □, subject D; ▲, subject E) and a 4-month-old infant (△). rPAc from transformant *S. mutans* TK18 was used as a coating antigen. Twofold serial dilutions of serum and saliva samples were assayed in triplicate by ELISA. The antibodies in the serum and saliva samples were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) and alkaline phosphatase-conjugated goat anti-human IgA(α), respectively. The data are expressed as means for triplicate assays.

the A-region of the PAc (residues 269 to 278, 289 to 298, 319 to 328, 339 to 348, and 399 to 408) and four decapeptides corresponding to the middle region (residues 809 to 818, 819 to 828, 829 to 838, and 839 to 848). The epitope-scanning patterns in the serum samples from the other four adult donors were similar to the pattern in the serum sample from subject A, and the ELISA titers in the epitope scanning corresponded to the serum antibody titers to rPAc (Fig. 2A and 3). The serum from a 4-month-old infant hardly reacted with any decapeptides. Figure 4 shows the results of epitope scanning with the saliva samples (1:10 dilution) from five adult donors. The epitope-scanning patterns in the saliva samples from five adult donors were similar to those in serum samples from the same donors, that is, salivary antibodies reacted strongly with the decapeptides that the serum antibodies gave positive reactions to but reacted weakly with the decapeptides that the serum antibodies gave a negative reaction to. However, the ELISA titers of saliva antibodies to these decapeptides did not correspond to the ELISA titers of serum antibodies to them. For example, in the serum from subject A the ELISA titers of the antibodies to the decapeptides were generally high while in the saliva of the same subject the titers of the antibodies to them were low. Antibodies in the saliva sample (1:10 dilution) from subject A showed strong reactions (A_{405} > 1.0) with the 14 decapeptides (residues 999 to 1008, 1059 to 1068, 1079 to 1088, 1129 to 1138, 1199 to 1208, 1249 to 1258, 1259 to 1268, 1289 to 1298, 1299 to 1308, 1329 to 1338, 1409 to 1418, 1439 to 1448, 1459 to 1468, and 1556 to 1565) corresponding to the C-terminal region of the PAc, while antibodies in the saliva sample reacted weakly with decapeptides corresponding to the N-terminal and the middle regions of the PAc (Fig. 4A). On the other hand, in the saliva from subject E the ELISA titers of the antibodies to the decapeptides were generally high even though the antibodies in serum from the same subject reacted weakly with them.

Epitope scanning of the A-region and the middle region. As noted above, the epitope scanning of the PAc with human sera suggested that antigenic epitopes exist in the A-region and the middle region. Therefore, we synthesized a series of 121 overlapping decapeptides covering the A-region and a series of 21 overlapping decapeptides covering the middle region (residues 824 to 853) to determine the continuous antigenic epitopes in the regions. The serum (1:800 dilution) from subject A showed positive reactions (A_{405} > 1.0) with 27 decapeptides of the 121 overlapping decapeptides covering the A-region (Fig. 5). Among the five decapeptides in the A-region with which the serum showed positive reactions in the epitope scanning of the PAc, four decapeptides were recognized as antigenic epitopes in the epitope scanning of the A-region. Although residues 269 to 278 were not identified as an antigenic epitope in the epitope scanning of the A-region, in the serum from subject A the ELISA titer of antibody to the decapeptide was relatively high (A_{405} = 0.74) (Fig. 5A). All the decapeptides that showed positive reactions with human sera had either one or two of the five common sequences YQAXL, NADAKA, VQKAN, NNAKNA, and IKKRNA, where X is Asp, Glu, Lys, or Ala, suggesting that the antigenicity of the A-region might be determined by a few sequential B-cell epitopes. The epitope-scanning patterns with sera from other four adult donors and overlapping decapeptides corresponding to the A-region were similar to the pattern with the serum from subject A (Fig. 5). The serum from an infant did not react with any of the overlapping decapeptides corresponding to the A-region. The serum (1:1,600 dilution) from subject A reacted strongly (A_{405} > 0.4) with the 6 decapeptides of the 21 overlapping decapeptides covering the middle region of the PAc molecule (residues 824 to 853) (Fig. 6). These 6 decapeptides had one of the two common sequences KVTKEKP and VKPTAPTK.

Reactivity of human serum with purified 20-mer peptides. On the basis of the data in the epitope scanning of the PAc, we synthesized seven 20-mer peptides containing antigenic epitopes. All of the serum samples from five adult donors reacted with all the 20-mer peptides coated on the ELISA plates (Fig. 7). The serum from a 4-month-old infant did not react with any 20-mer peptides. To examine whether the antigenic activities of these peptides differ when they are tested as free peptides in solution or adsorbed to a layer of plastic, we used the inhibition assay for the binding of antibodies to rPAc. rPAc, PAc(96–114), PAc(331–349), PAc(513–531), PAc(623–641), PAc(824–842), and PAc(835–853) at 400 μg/ml resulted in 92.4% ± 3.6%, 23.6% ± 5.6%, 10.3% ± 2.0%, 23.3% ± 3.0%, 23.1% ± 0.9%, 31.8% ± 3.5%, and 19.7% ± 2.4%

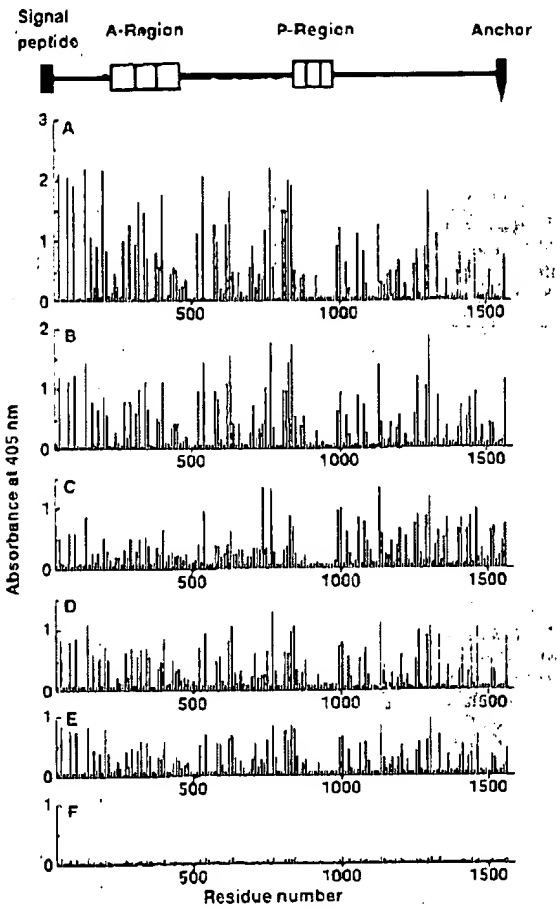

FIG. 3. Reactivities of human sera from five adult subjects (A to E) and a 4-month-old infant (F) with 153 decapeptides covering the complete amino acid sequence of the mature PAc. The reactivities of the peptides with human sera (1:800 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means of A_{405}s after 1 h of incubation with the substrate for six assays. The proposed model of PAc is shown at the top. The residue number corresponds to the position of the amino-terminal residue of the indicated peptide in the PAc sequence (39).

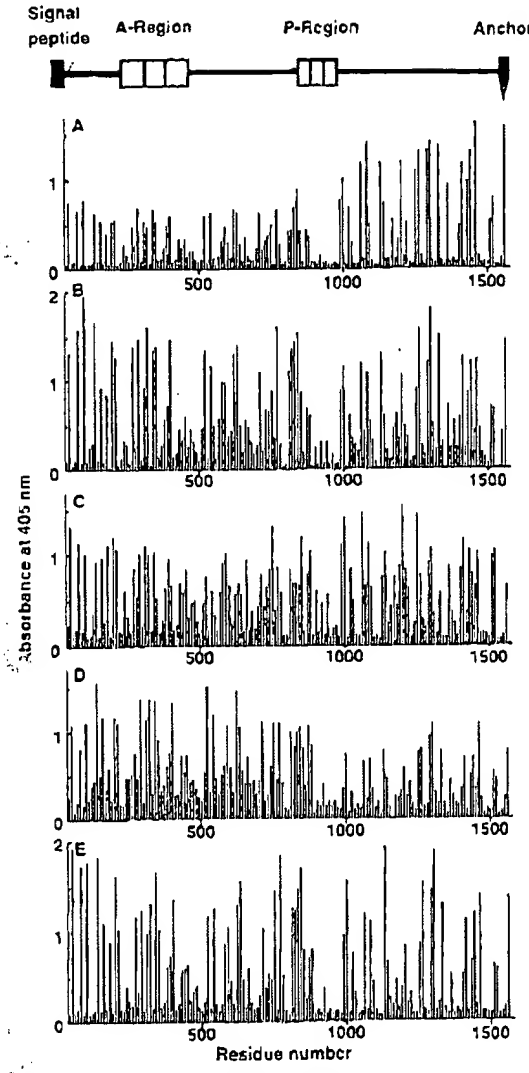

FIG. 4. Reactivities of human saliva from five adult subjects (A to E) with 153 decapeptides covering the complete amino acid sequence of the mature PAc. The reactivities of the peptides with human saliva (1:10 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgA(α). The data shown are means of A_{405}s after 1 h of incubation with the substrate for six assays. The residue number corresponds to the position of the amino-terminal residue of the indicated peptide in the PAc sequence (39).

(mean ± standard deviation) inhibition, respectively, for the binding of antibodies to rPAc. However, PAc(764–782) and an irrelevant peptide, NA(464–482), at 400 μg/ml had no inhibitory effect. The discrepancy in PAc(764–782) might be ascribed to the aggregation of the peptide in solution or to the difference in conformation between the solid phase and the solution.

DISCUSSION

Using a complete set of decapeptides covering the mature PAc molecule, Takahashi et al. (47) mapped the antigenic sites of the protein antigen molecule in various strains of mice. They showed that the antigenic epitopes are scattered throughout the molecule and also that the antigenic epitope patterns differ in mice with different *H-2* haplotypes. To identify continuous antigenic epitopes in humans, we synthesized 153 decapeptides covering the mature PAc, 121 overlapping decapeptides covering the A-region of the PAc, and 21 overlapping decapeptides covering the middle region (residues 824 to 853) according to the multiple pin-coupled peptide technology. The antigenic epitope patterns that were determined by using these decapeptides and samples of serum and saliva from human subjects harboring *S. mutans* in their oral cavity differed considerably from those in mice. Despite the difference of the HLA type among these subjects, the epitope patterns in humans were similar to each other.

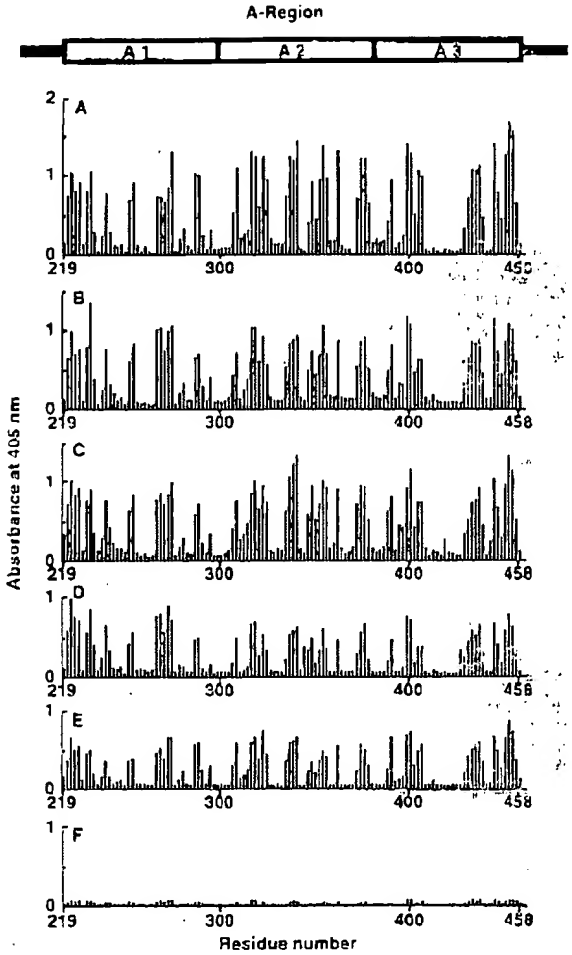

FIG. 5. Reactivities of human sera from five adult subjects (A to E) and a 4-month-old infant (F) with 121 overlapping decapeptides covering the A-region of the PAc protein. The reactivities of the peptides with human sera (1:800 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means of A_{405}s after 1 h of incubation with the substrate for three assays. The residue number corresponds to the position of the amino-terminal residue of the indicated peptides in the PAc sequence (39).

In this study, 27 decapeptides of the 121 overlapping decapeptides covering the A-region showed positive reactions with human sera. All of these 27 decapeptides had one or two of five common sequences consisting of 4 to 6 residues, suggesting that the antigenicity of the A-region might be determined by a small number of B-cell epitopes. It is, however, difficult to rule out the presence to other B-cell epitopes, because only a limited number out of all possible epitopes were sampled in this study.

The PAc protein of *S. mutans* binds to human salivary agglutinin (3, 10, 43). The binding of the cell surface antigen to salivary agglutinin is considered to play an important role in the in vivo colonization of agglutinin-coated tooth surfaces by *S. mutans* (22, 26). Recently, several investigators have reported that a salivary agglutinin-binding domain exists in the A-region of the PAc (P1, SR) of *S. mutans* (9, 33, 35). Furthermore, it has been suggested that the A-region of the streptococcal cell surface antigen has antigenic and immunogenic epitopes in mice and rabbits (33, 40, 48). Takahashi et al.

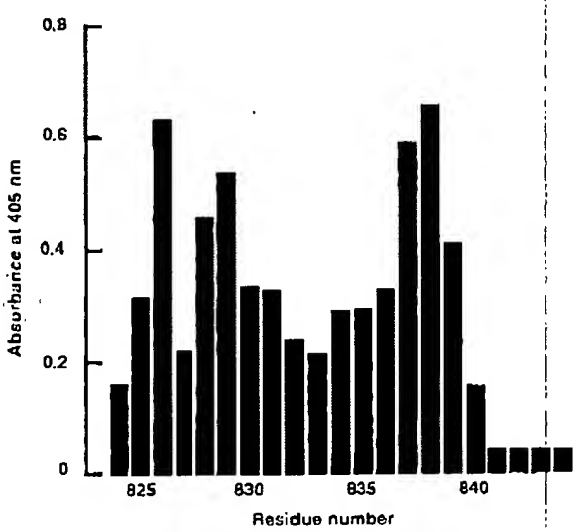

FIG. 6. Reactivities of human serum from subject A with 21 overlapping decapeptides covering the middle region of the PAc protein (residues 824 to 853). The reactivities of the peptides with human sera (1:1,600 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means of A_{405}s after 1 h of incubation with the substrate for three assays. The residue number corresponds to the position of the amino-terminal residue of the indicated peptides in the PAc sequence (39).

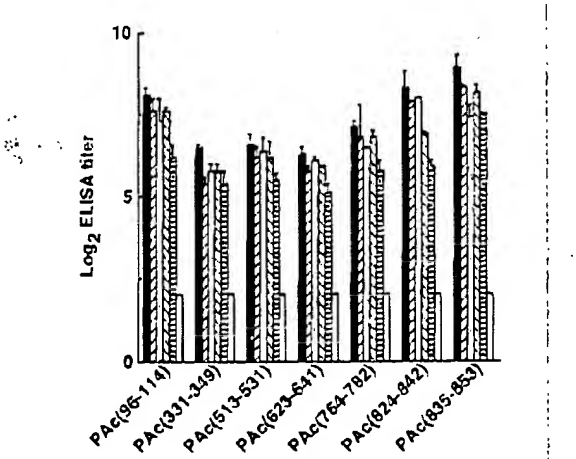

FIG. 7. Serum antibody responses to 20-mer synthetic peptides in five adult subjects (■, subject A; ▨, subject B; □, subject C; ▩, subject D; ▣, subject E) and a 4-month-old infant (▯). The binding of the antibodies to synthetic peptides was detected by ELISA using alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means ± standard deviations of the $\log_2$ ELISA antibody titers for triplicate assays. All experiments were performed three times, and similar results were obtained in each experiment.

(48) reported that the peptide ANAANEADYQAKLTAY QTE, corresponding to residues 301 to 319 of the PAc protein [PAc(301–319)], reacts with murine antisera to the PAc protein and induces protective immune responses in BALB/c mice. The peptide PAc(301–319) has one (YQAKL) of five amino acid sequences common among A-region-corresponding decapeptides that showed positive reactions with sera from human subjects harboring *S. mutans* in their oral cavity. Okahashi et al. (40) have identified antigenic epitopes in the A-region using antisera to rPAc from BALB/c, B10, B10.D2 and B10.DR mice and overlapping decapeptides covering the second repetitive unit of the A-region. They found that the peptide YEAALKQY (residues 366 to 373) is recognized by anti-rPAc sera from all of four strains of mice. In this study, the peptide KATYEAALKQ (residues 363 to 372) showed a positive reaction with human sera. Recently, Moisset et al. (33) have synthesized the peptide TELARVQKANADAKAAY (peptide 2), corresponding to the first repetitive unit of the A-region of the SR protein of serotype f *S. mutans*. Peptide 2 had two sequences, VQKAN and NADAKA, of the five antigenic common epitopes identified in this study. They demonstrated that peptide 2 reacts with anti-SR rabbit IgG and that the subcutaneous immunization of rabbits with the peptide conjugated to ovalbumin induces antibodies reactive with the peptide and SR protein. In addition, they showed that peptide 2 is able to inhibit the binding of human salivary glycoproteins to the SR protein. Taken together, these results suggest that the A-region of the PAc protein is a potentially important antigenic and functional domain.

PAc (I/II) of *S. mutans* is known to serologically cross-react with SpaA (PAg) of *S. sobrinus* (18, 42, 45, 49). The homology between the two protein antigens is 66% (25, 51). The sequences YEAKL and NAQAKA, which are similar to the antigenic common amino acid sequences in the A-region of PAc, are present in the amino acid sequence of the PAg (51). Recently, Okahashi et al. (40) demonstrated that residues 362 to 373 of the PAc protein and residues 365 to 376 of the PAg protein are one of the cross-reactive epitopes of both protein antigens in BALB/c mice. In this study, residues 363 to 372 of the PAc were identified as an antigenic epitope in humans. In addition, a saliva-binding protein (SSP-5) of *Streptococcus sanguis*, which is isolated most frequently among oral streptococci from human tooth surfaces, has a 59% homology with PAc of *S. mutans*, and the structure of the protein is very similar to that of PAc (11). The sequences NADAKA, VQ KAN, and NNAKNA exist in the amino acid sequence of the SSP-5 (11). Amino acid sequence homology research (National Biomedical Research Foundation-Protein Data Base, Washington, D.C.) showed that the antigenic common sequences YQAEL, YQADL, VQKAN, NADAKA, NNAKNA, and IKKRNA in antigenic epitopes in the A-region of PAc (I/II) exist in the amino acid sequences of 9, 7, 2, 32, 0, and 3 other proteins, respectively. It is possible that some of the antibodies to antigenic epitopes in the PAc might result from either cross-reactions or epitopes shared with other proteins if humans are exposed to them (50).

In the epitope scanning of the PAc protein with human saliva and serum samples, the reactivities against four decapeptides corresponding to the middle region of the PAc (residues 809 to 848) were as great as the reactivities against decapeptides corresponding to the A-region (Fig. 3 and 4). In addition, the reactivities of two 20-mer peptides, PAc(824–842) and PAc(835–853), with serum antibodies were greater than those of other 20-mer peptides (Fig. 7). Therefore, we synthesized 21 overlapping decapeptides covering the middle region (residues 824 to 853). Of the 21 decapeptides, 6 decapeptides reacted strongly with serum antibodies from subject A. Each of the six decapeptides had one of the two common sequences KVT KEKP and VKPTAPTK. The sequence KVTKEKP is present in PAg of *S. sobrinus* (residues 833 to 839) (51). In addition, residues 809 to 849 of the PAc adjacent to the P-region (residues 851 to 967) are identical to residues 813 to 843 of the PAg (39, 51). These findings suggest that the middle region adjacent to the P-region may be one of the immunologically cross-reactive domains of the *S. mutans* PAc protein and the *S. sobrinus* PAg protein.

Western blotting analysis with human sera demonstrated that the PAc protein of *S. mutans* was immunodominant in humans, in agreement with the results of other investigators (7, 44, 50). It has been reported that serum IgG antibodies to the immunodominant antigen I/II (PAc) are significantly greater in subjects with an experience of low caries than in subjects with high caries, thus suggesting that naturally induced serum IgG antibodies to the PAc are associated with protection against dental caries (7). Moreover, Challacombe (6) showed that there is an inverse relationship between the serum IgG antibodies to *S. mutans* and salivary IgA antibodies, and changes in the salivary antibody titer are negatively correlated with changes in the serum IgG antibody titer. These findings were also confirmed by the present study. Titers of salivary IgA to rPAc and synthetic peptides were not correlated with titers of serum antibody to them in this study. The inverse relationship between the serum IgG antibodies to *S. mutans* antigens and salivary IgA antibodies is therefore considered to be worthy of future study.

It has been thought that hydrophobic amino acids tend to be buried within the native structure of globular proteins, while hydrophilic side chains are on the exterior where they can interact with water (52). Hopp and Woods (19) showed that most hydrophilic segments of a protein tend to correspond to continuous antigenic epitopes. In this study, we predicted the hydrophilicity of the PAc molecule according to the procedure of Kyte and Doolittle (23). All the decapeptides that showed positive reactions with human sera had hydrophilic segments, but the chi-square statistical analysis showed that there was no correlation between antigenicity and hydrophilicity. Geysen et al. (14) also reported that there is little correlation between antigenicity and hydrophilicity in a myohemerythrin protein. Moreover, we predicted the secondary structure of the PAc molecule according to the procedure of Chou and Fasman (8). All the antigenic epitopes in the C-terminal three-fourths of the PAc protein contained beta-turn populations, but only three antigenic epitopes in the N-terminal quarter, which was predicted to be totally alpha-helical (39), contained beta-turn populations. These results suggest that antigenic epitopes may be located in the alpha-helixes at the surface of the protein as well as in the beta-turn populations.

In conclusion, we identified the continuous antigenic epitopes in the PAc molecule using 295 decapeptides and seven 20-mer synthetic peptides and human sera and saliva from subjects infected naturally with *S. mutans*. The identification of these immunodominant antigenic epitopes is considered to be a first step in the development either of specific diagnostic tests or of a vaccine against human dental caries.

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T-Cell, Adhesion, and B-Cell Epitopes of the Cell Surface *Streptococcus mutans* Protein Antigen I/II

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The T-cell and antibody responses to a cell surface streptococcal antigen (SA I/II) were investigated in naturally sensitized humans. Serum antibody responses were directed predominantly to the N-terminal (residues 39 to 481) and central (residues 816 to 1213) regions of SA I/II which may be involved in bacterial adhesion to salivary receptors. T-cell responses were also directed predominantly towards the central region. The linear peptide relationship of the immunodominant and minor T- and B-cell as well as adhesion epitopes was mapped within residues 816 to 1213. Immunodominant T-cell and B-cell epitopes were identified within residues 803 to 853, which were separated in linear sequence from the adhesion epitopes (residues 1005 to 1044). Adhesion epitopes overlapped with minor B- and T-cell epitopes (residues 1005 to 1054 and 1085 to 1134). An immunodominant promiscuous T-cell epitope (residues 985 to 1004) was adjacent to an adhesion epitope (residues 1005 to 1024). The limited B-cell response to adhesion epitopes is consistent with the success of *Streptococcus mutans* in colonizing the oral cavity. The strategy of T-cell, adhesion, and B-cell epitope mapping has revealed a general approach for identifying components of subunit vaccines which may focus responses to critical functional determinants. Such epitopes of SA I/II may constitute the components of a subunit vaccine against dental caries.

Active immunization with whole cells of *Streptococcus mutans* prevents colonization of the tooth surface by this microorganism and the development of dental caries in animal models (4, 25, 35, 51). Similar protection against caries is induced in nonhuman primates by systemic immunization with a cell surface protein with an M_r of 185,000 (27), variously termed streptococcal antigen I/II (SA I/II) (42, 43), antigen B (45), P1 (15), or PAc (39). Immunization with SA I/II elicits serum immunoglobulin G (IgG), IgA, and IgM antibodies as well as $CD4^+$ T-cell proliferative responses (22, 23). Passive systemic immunization with the IgG class of antibodies raised against *S. mutans* also prevents caries in nonhuman primates (28), indicating a role for serum antibodies in protection. Serum IgG[1] passes to gingival fluid and thus has direct access to the tooth surface (6, 49). The importance of antibody in protection was further demonstrated by topical application to the tooth surface of monoclonal antibodies (MAbs), raised against SA I/II, which prevented colonization with *S. mutans* and the development of caries in nonhuman primates (24). In humans, the same procedure conferred long-term protection against colonization with *S. mutans* (30, 32).

SA I/II may function as an adhesin which mediates attachment of *S. mutans* to the tooth surface since it is essential for colonization in vivo (24, 32) and binds to salivary receptors in vitro (13, 44). The gene encoding SA I/II from two strains of *S. mutans*, serotype c, has been sequenced (19, 40). The deduced amino acid sequence of SA I/II from *S. mutans* NG5 (19) comprises 1,561 residues and includes a signal peptide (residues 1 to 38) and a predicted C-terminal bacterial cell wall-spanning region, as well as two series of tandemly repeated sequences. There are four repeats of an alanine-rich 82-residue sequence (residues 121 to 447) and three repeats of a proline-rich 39-residue sequence (residues 839 to 955). Sequencing of homologous genes from other streptococci (12, 21, 38, 52) as well as hybridization studies (31) indicates that SA I/II is a member of a gene family conserved among oral streptococci.

At least two distinct sites in SA I/II interact with salivary receptors in vitro. These are within residues 816 to 1213, the most conserved portion of SA I/II (37), and within residues 186 to 469, the alanine-rich sequence (10). Both MAbs which prevent colonization with *S. mutans* in humans (32) recognize epitopes within residues 816 to 1213 (37). In addition, immunization with a synthetic peptide derived from the alanine-rich region of SA I/II from *S. mutans* MT8148 (residues 301 to 319) suppressed colonization of murine teeth by *S. mutans* (50). Taken together, these observations suggest that protection against dental caries may be conferred by the induction of serum antibodies which recognize receptor-binding regions of SA I/II.

Limited data are available regarding epitopes of SA I/II recognized by naturally sensitized humans. Serum and salivary responses to a panel of nonoverlapping decapeptides which spanned the complete sequence of SA I/II were determined for five subjects (34). Peptides throughout the sequence were recognized although serum antibodies showed some preference for peptides in the N-terminal and middle regions. Salivary IgA antibodies showed similar recognition but with higher titers to peptides within the C-terminal region.

In this study, we have investigated human T- and B-cell responses to recombinant polypeptide fragments which span the extracellular portion of SA I/II (37). The positions of these fragments within SA I/II are shown in Fig. 6, which also summarizes data on adhesion binding sequences. These analyses indicated that the fragment corresponding to residues 816 to 1213 is an immunodominant region of SA I/II. We have inves-

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tigated the relationship among T-cell, adhesion, and B-cell epitopes by using a panel of synthetic peptides to map epitopes within this region. We have previously used the term EPITAB mapping for this procedure (26) and propose it as a general procedure for identifying components of a subunit vaccine.

MATERIALS AND METHODS

Materials. Fmoc amino acids, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), and Rink amide 4-methyl-benzhydrylamine (MBHA) resin were purchased from Calbiochem-Novabiochem (UK) Ltd. (Nottingham, United Kingdom). Dimethylformamide, trifluoroacetic acid, diethyl ether, dichloromethane, and piperidine were purchased from Romil Chemicals Ltd. (Loughborough, United Kingdom). Diisopropylethylamine was from Aldrich Chemical Co. (Poole, Dorset, United Kingdom). Oligonucleotides were purchased from Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom).

Subjects. The caries index (decayed, missing, and filled surfaces [DMFS]) of 50 healthy volunteers from the Department of Immunology was determined, and venous blood and saliva samples were collected. HLA-DR typing of 17 subjects was performed by the Tissue Typing Department (Guy's Hospital).

Bacteria and growth conditions. To prepare SA I/II, *S. mutans* Guy's strain (serotype c) was grown in 10 liters of supplemented basal medium, as described previously (6, 7), at 37°C, for 72 h. For the adhesion assay, *S. mutans* was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). *Escherichia coli* BL21 (DE3) (Novagen Inc., Madison, Wis.) harboring pET15b was grown at 37°C in Luria-Bertani broth supplemented with carbenicillin (50 μg/ml), and recombinant protein expression was induced with isopropyl-β-D-thiogalactopyranoside (1 mM) (37).

Antigens. SA I/II was prepared from *S. mutans* Guy's strain (serotype c) as described previously (42). Recombinant fragments 1 (residues 39 to 581), 2 (residues 475 to 824), and 4 (residues 1155 to 1538) expressed in *E. coli* BL21 by using pET15b were purified by affinity chromatography as described previously (22). For this study, the portion of the gene encoding residues 816 to 1213 (fragment 3) was amplified by PCR with the plasmid pSM I/II as template with the oligonucleotide primers (5′) ttacatatgAATGGTAAAATCCGTGCCGTT, the sequence in uppercase letters corresponding to nucleotides 2442 to 2462 of the SA I/II gene, and (3′) ctggatccctcCACTA AATCCTGACGCAATTCAAG, the sequence in uppercase letters being complementary to nucleotides 3636 to 3659. The amplified fragment was cloned into the vector pET15b and expressed in *E. coli* BL21 (DE3) exactly as described previously (37). Similarly, the portion of the gene encoding residues 984 to 1161 was amplified by using the oligonucleotide primers (5′) atatcatgCCAACTGTTCATTTCCATTACTTT (nucleotides 2949 to 2972) and (3′) gcaattgctcga ctcaTTCATTTTTATTAACCTTAGT (complementary to nucleotides 3462 to 3482). The amplified fragment was cloned into pET15b modified by the addition of a *Sal*I site (37). The recombinant polypeptide was purified by affinity chromatography on Ni^{2+} affinity resin as described previously (37). All antigens were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Gels were scanned with the GS 670 imaging densitometer (Bio-Rad Laboratories, Richmond, Calif.).

Synthetic peptides. Peptide amides (20-mers overlapping by 10 residues) were synthesized on Rink amide MBHA resin in sealed porous polypropylene bags by the manual simultaneous multiple peptide synthesis procedure (18) with Fmoc chemistry. PyBOP was used as coupling agent, and Fmoc-amino acids were activated in situ by addition of diisopropylethylamine. Following 20 cycles of synthesis, resin was washed with dimethylformamide followed by dichloromethane and peptides were cleaved by incubation in trifluoroacetic acid-ethanedithiol-anisole-phenol-H_2O (82.5:2.5:5:5:5 [vol/vol/vol/wt/vol]) for 2 h at room temperature. Peptides were precipitated by the addition of 5 volumes of ether, recovered by centrifugation, and washed three times with ether. Finally, peptides were dissolved in water and lyophilized. The scale of synthesis was 50 μmol. Aliquots of each peptide were hydrolyzed in 6 M HCl at 110°C for 24 h, and compositions were determined by S. Lachwell (National Institute for Medical Research, Mill Hill, London, United Kingdom) with the Beckman 121MB automated analyzer (Beckman Instruments Ltd., High Wycombe, United Kingdom). In each case, the composition matched that predicted.

Antibodies. MAbs L243 (anti-major histocompatibility complex [MHC] class II) and W6/32 (anti-MHC class I) were produced from cultures of hybridomas obtained from the American Type Culture Collection (Rockville, Md.). ID4, an isotype (IgG2a)-matched control of irrelevant specificity, was provided by P. Shepherd (Department of Immunology, United Medical and Dental Schools). Rabbit anti-SA I/II antiserum was prepared as described previously (42).

Lymphoproliferative assay. Defibrinated blood from volunteers was separated on a Ficoll gradient. Sera were used for antibody assays (see below) while peripheral blood mononuclear cells (PBMCs) were washed and resuspended in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.), supplemented with 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), and 10% heat-inactivated autologous serum. PBMCs (10^5 cells per well) were cultured in 96-well round-bottomed plates (Costar, Cambridge, Mass.) in a total volume of 200 μl. Three replicates of each culture were incubated with three concentrations (1, 10, and 40 μg/ml) of SA I/II, recombinant fragments, nonrecombinant control, or synthetic peptides. Incubation was at 37°C in a humidified atmosphere with 5% CO_2 for 6 days. Each culture received 0.2 μCi (7.4 kBq) of [^{3}H]thymidine (Amersham International, Little Chalfont, United Kingdom) 6 h before harvesting. Cultures were harvested onto glass fiber filters with a Dynatech (Chantilly, Va.) Minimal Cell harvester, and [^{3}H]thymidine incorporation was measured with the LKB liquid scintillation counter (LKB, Bromma, Sweden). Proliferation was expressed as stimulation index (SI), which is the mean counts per minute of antigen-stimulated cultures divided by counts per minute of antigen-free cultures. Concanavalin A (10 μg/ml) (Sigma Chemical Co.) was used with every culture as a positive control, but the results are not presented.

MHC dependency of proliferative responses to SA I/II was determined by culturing lymphocytes with antigen (10 μg/ml) as described above in the presence of MAb L243, W6/32, or ID4 at 1, 10, and 20 μg/ml. Cultures were incubated with [^{3}H]thymidine and harvested, and [^{3}H]thymidine uptake was determined as described above.

ELISA for serum antibodies. Antibody recognition of SA I/II, recombinant fragments, and synthetic peptides was determined by enzyme-linked immunosorbent assay (ELISA). Proteins (2 μg/ml) or peptides (10 μg/ml), in phosphate-buffered saline (PBS) were adsorbed to wells of polystyrene microtiter plates (Dynatech) for 2 h at room temperature. Plates were washed, and wells were treated with 1.5% (wt/vol) bovine serum albumin (BSA) for 1 h at room temperature to block unbound sites. After washing, bound antigens were incubated with serially diluted sera in duplicate. Bound IgG antibodies were determined by incubation with alkaline phosphate-conjugated goat anti-human Ig (Sigma Chemical Co.) and subsequent reaction with *para*-nitrophenyl phosphate (Sigma Chemical Co.). Plates were read at 405 nm with the microplate reader model 450 (Bio-Rad). Three assays were performed with the recombinant fragments. For the peptides, after initial screening with the complete panel the assay was repeated at least three times with each serum with a restricted set of peptides. SA I/II (2 μg/ml) was included in each assay as was an irrelevant peptide (HQAAM HQIRDIINFEAADWD) derived from the sequence of simian immunodeficiency virus p27. For all assays, results are expressed as the highest dilution giving an absorbance value of ≥0.2.

Western blotting (immunoblotting). Serum antibody responses were also assayed by Western blotting with SA I/II, the recombinant polypeptides, and a control fraction from *E. coli* BL21 harboring nonrecombinant pET15b. Purified antigens were separated by SDS-PAGE with gels of 10% acrylamide, by using a minigel system (Hoefer Scientific Instruments, San Francisco, Calif.). Proteins were transferred to nitrocellulose with a semidry blotter (Sartorius A.G., Gottingen, Germany). Nitrocellulose strips were blocked with 5% (wt/vol) nonfat milk powder–2.5% (wt/vol) BSA in Tris-HCl-buffered saline (pH 8.0) containing 0.05% (wt/vol) Tween 20. Strips were subsequently incubated with human sera (1 in 20 dilution) or rabbit anti-SA I/II antiserum (10^{-3} dilution), and bound antibody was visualized by using alkaline phosphatase-conjugated secondary antibody with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma Chemical Co.) as substrates. Each serum was assayed three times, and responses were considered as positive if bands were visible in at least two assays.

Bacterial adherence assay. SA I/II-mediated adherence of *S. mutans* (Guy's strain) to saliva was assayed by determining binding of [^{3}H]thymidine-labelled bacteria to saliva adsorbed to microtiter wells. Freshly collected human saliva from a single donor was clarified by centrifugation for 10 min at 3,000 × *g*, heat inactivated at 60°C for 30 min, and finally clarified by centrifugation at 17,000 × *g* for 10 min. Treated saliva was diluted with an equal volume of PBS and adsorbed to the wells of a polystyrene 96-well flat-bottomed microtiter plate (Immulon 4, Dynatech) for 2 h at room temperature. After coating, wells were washed three times with PBS and unbound sites were blocked by incubation with 1.5% (wt/vol) BSA in PBS for 1 h at room temperature. Plates were then washed three times with 50 mM KCl–1 mM $CaCl_2$–38 mM $MgCl_2$–1 mM KH_2PO_4–1.2 mM K_2HPO_4 (pH 7.2) (adherence buffer) (9). *S. mutans* cells from an overnight culture in Todd-Hewitt broth were used to inoculate (1/10 volume) a further culture in Todd-Hewitt broth containing 100 μCi (3.7 MBq) of [^{3}H]thymidine (Amersham International plc) per ml. Cells were harvested in mid- to late log phase (optical density at 700 nm of ≤0.4), pelleted by centrifugation at 1,000 × *g* for 10 min, and washed three times in adherence buffer. The final suspension was vortexed with 0.5 volume of glass beads to break up chains of cocci which were monitored microscopically (37). Cells were resuspended to give 5 × 10^6 cpm/50 μl, and BSA was added to 1.5% (wt/vol). Specific activity of the washed *S. mutans* cells was estimated to be 1.0 × 10^{-3} to 1.3 × 10^{-3} cpm per cell (37). In competitive inhibition of adherence, native SA I/II, fragment 1, fragment 3, recombinant 984-1161, and myoglobin were added to the wells (at final concentrations of 0.5 to 5 μM) in 50 μl of adherence buffer containing 1.5% (wt/vol) BSA together with 50 μl of radiolabelled *S. mutans* suspension. The plates were incubated at 37°C for 2 h with gentle shaking and subsequently were washed 10 times with adherence buffer. Bound *S. mutans* cells were eluted with 1% (wt/vol) SDS and transferred to glass fiber filters by using the Micromate 196 cell harvester (Canberra Packard, Pangbourne, United Kingdom). Filters were counted with the Matrix 96 direct beta counter (Canberra Packard). Background binding was determined on wells to which no saliva was adsorbed. Synthetic peptides (concentration, 62.5 to 500 μM) were assayed for inhibition of adhesion by the same procedure. The percentage of binding of *S. mutans* to saliva was calculated

TABLE 1. T-cell and serum responses to SA I/II and recombinant fragments[a]

Antigen	T-cell response		Antibody response	
	SI (± SEM)	Frequency	$\log_2$ (titer)	Frequency
SA I/II	11.6 ± 2.6	17/18	9.1 ± 0.27	18/18
Fragment				
1	10.7 ± 3.2	2/18	4.27 ± 0.29	2/10
2	6.5 ± 1.2	1/18	4.6 ± 0.34	5/18
3	22.6 ± 4.9	9/18	4.9 ± 0.22	11/18
4	10.3 ± 2.9	4/18	4.1 ± 0.31	5/18
Nonrecombinant plasmid	6.1 ± 1.1	4/18	3.0 ± 0.24	0/18

[a] Mean SI (± SEM) of all subjects (n = 18) is presented for SA I/II and each fragment. The frequency of positive responses is shown. Positive responses to SA I/II were those with SI of ≥3.0 (>500 cpm). Positive responses to the fragments were those with SI greater than mean + 2 SD of the response to the control protein fraction from *E. coli* harboring nonrecombinant plasmid. With medium only, the response was 561 ± 104 cpm. Serum responses (determined by ELISA) are shown as mean $\log_2$ (titer) (± SEM) for all subjects. Positive responses were those with a titer greater than mean + 2 SD of the response to the control protein fraction (nonrecombinant plasmid).

by the formula [(test counts per minute) − (control counts per minute)/total counts per minute] × 100. Percent inhibition of adherence was calculated as [(percent adherence without inhibitor − percent adherence with inhibitor)/percent adherence without inhibitor] × 100. For protein, determinations of streptococcal adhesion were made in triplicate or quadruplicate at each protein concentration, while for peptides, duplicate determinations were made. In each case, the assay was performed at least three times.

Statistics. The Student t test was used to analyze the results.

RESULTS

Lymphoproliferative response to SA I/II and recombinant fragments. SA I/II and the recombinant polypeptides used in this study were purified and analyzed by SDS-PAGE and were documented in a previous paper (37). The purity of the polypeptides was estimated by densitometric scanning to be >85%. Some bands with a lower M_r than those of the intact polypeptides were evident. Since these bands were recognized by rabbit anti-SA I/II antiserum and by MAbs (37), they may be products of partial proteolysis. Proliferative responses of PBMCs from 18 subjects were determined following in vitro stimulation with SA I/II and the recombinant fragments spanning the predicted extracellular portion of SA I/II. Stimulation with concanavalin A induced proliferation in each experiment with mean SI for the population being 49 ± 9.2 with a range of 14.7 to 146.5 (result not shown). All but one of the individuals responded significantly to SA I/II with an SI of >3 at doses of 1 to 40 μg/ml, with peak responses generally at 10 μg/ml (approximately 50 nM) and mean SI for the population being 11.6 ± 2.6 (Table 1). Since each recombinant fragment preparation will include trace components derived from *E. coli* which may themselves induce proliferative responses, a protein fraction was prepared from *E. coli* harboring nonrecombinant pET15b by exactly the same procedure as that used to purify the recombinant fragments. This fraction was used as a background control for responses to the fragments (but not for those to SA I/II). The responses to each fragment are also shown in Table 1 as the mean SI (± standard error of the mean [SEM]) of all 18 subjects. Among the four recombinant fragments, only fragment 3 stimulated a significantly greater response ($P < 0.005$) than the control protein fraction from *E. coli* harboring the nonrecombinant plasmid (Table 1). Individual responses to the fragments were considered positive if the SI was greater than the mean + 2 standard deviations (SD) of the response to the nonrecombinant control. The highest frequency of such responses was to fragment 3, for which the responses of 9 of 18 subjects had an SI greater than the mean + 2 SD of the responses to the control (Table 1).

T-cell epitope mapping. As significant lymphoproliferative responses were generated only with fragment 3, a panel of 32 overlapping synthetic peptides, spanning residues 803 to 1174, was prepared. Proliferative responses of PBMCs from 30 subjects were determined by stimulation with the peptides. All subjects responded to at least one peptide, with a band range of 1 to 8 peptides and a mean of 4.4 peptides. On the basis of frequency of response to each peptide (SI ≥ 3.0, >500 cpm), three immunodominant epitopes were identified: peptides 803-822, 975-994, and 985-1004, each yielding frequencies of >50% (Fig. 1). Since most (13 of 15) subjects who responded to peptide 975-994 also responded to peptide 985-1004, it is probable that a single T-cell epitope is present within residues 975 to 1004. Minor T-cell epitopes were also identified within peptides 1005-1024, 1015-1034, 1085-1104, and 1115-1134, with frequencies of >20%, and some of the adjacent peptides may represent single T-cell epitopes. However, eight other peptides with frequencies of >10 and <20% may also function as minor T-cell epitopes, but they were not studied further.

MHC restriction of the lymphoproliferative responses. HLA restriction of the T-cell response was first studied by dose-dependent inhibition with MAb to HLA class I and II antigens (Fig. 2). The lymphoproliferative response was inhibited by 50% with 1 μg of MAb to HLA-DR (L-243), and 10 μg of the MAb inhibited 100% of the responses (from SI of 10.0 ± 3.2 to SI of 1.5 ± 0.4). Neither MAb to HLA class I (W6/32) nor the isotype control induced any inhibition of the lymphoproliferative response.

The HLA-DRs of 17 subjects were determined, and six of these were homozygous. The responses to the immunodominant and minor epitopes were then studied in the six DR homologous subjects (Table 2). Only peptide 975-994 appeared to be restricted by HLA-DR1. The other six peptides stimulated lymphocytes from HLA-DR1, -2 (except peptide 1085-1104), and -6 (except peptide 803-822). DR5 was restricted by peptide 803-822, though the latter stimulated lymphocytes with DR1, -2, and -3 antigens. Lymphocytes with DR3 or -4 antigen responded to three or four peptides. The results suggest that, except for peptide 975-994, the remaining six peptides appear to be promiscuous as they stimulated lymphocytes with three to five HLA-DR antigens.

B-cell epitope mapping. Serum responses were determined by ELISA for the 18 subjects whose proliferative responses to the fragments were analyzed as described above, and all subjects showed significant recognition of SA I/II (Table 1). The recombinant fragments were recognized with lower titers than SA I/II, although the mean titer for each fragment (all subjects, n = 18) was significantly greater than that of the nonrecombinant control ($P < 0.01$) (Table 1). The frequency of responses (i.e., those greater than mean + 2 SD of the control) to fragment 3 was higher than those to the remaining fragments.

Recognition of SA I/II and the recombinant fragments was also analyzed by Western blotting with the same set of sera as in the ELISA as well as sera from four further subjects. A different pattern of recognition was evident in which both fragments 1 and 3 were most frequently recognized. The recombinant polypeptide corresponding to residues 984 to 1161 which represents a truncated form of fragment 3, lacking the tandem repeats of the proline-rich sequence as well as C-terminal residues, was also analyzed. From analyses of all 22 subjects, three main patterns of recognition were evident as shown in Fig. 3. Most subjects recognised SA I/II, fragments 1 and 3, and the truncated form of fragment 3 strongly together

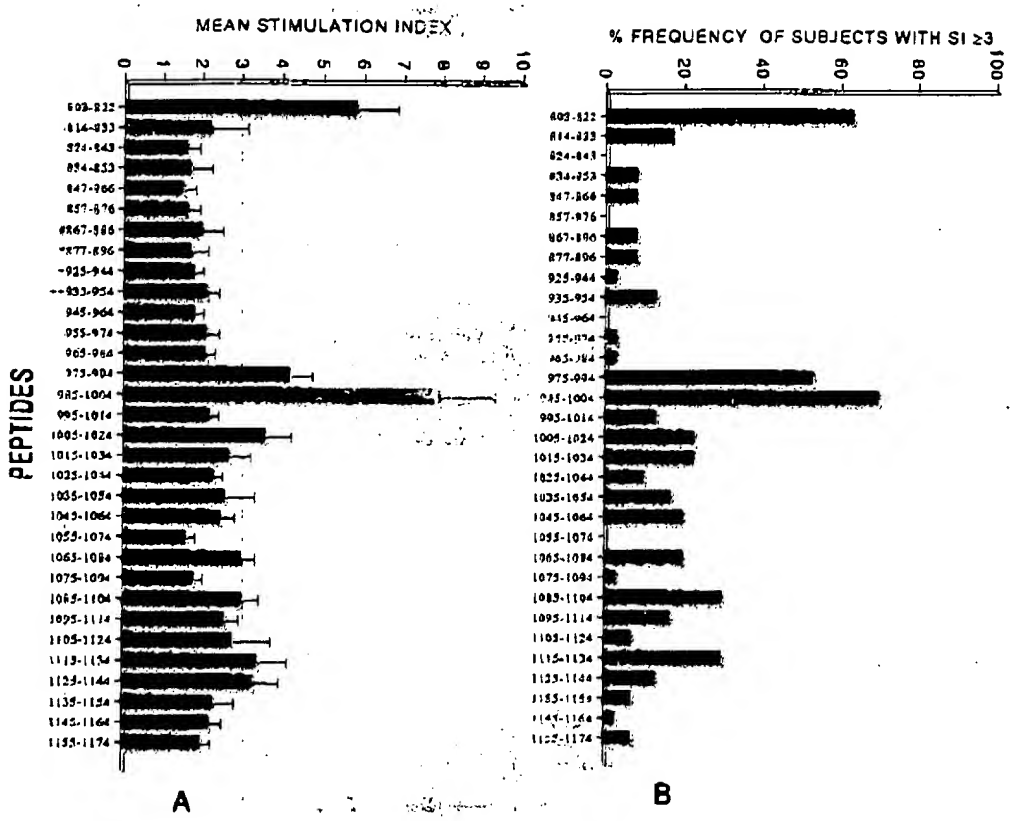

FIG. 1. Proliferative responses to overlapping synthetic peptides (20-mers) of SA I/II. (a) Mean SI (± SEM) of PBMCs from 30 subjects. Mean counts per minute with medium only were 538 ± 112. (b) Frequency of positive responses (SI, ≥3 [i.e., >500 cpm]). #, peptide 867-886 has the same sequence as peptide 906-925. Similarly, peptides 877-886* and 916-935 have identical sequences as do peptides 886-905 and 925-944+ and peptides 896-915 and 935-954++.

with weaker recognition of fragment 2 (panel a). A smaller proportion showed weak recognition of SA I/II and of fragment 3 only (panel b) or strong recognition of SA I/II with weak recognition of one or more fragments (panel c). Rabbit anti-SA I/II antiserum used as a positive control (panel d) recognized fragments 1 to 4 strongly, as well as the recombinant polypeptide 984-1161 more weakly. No recognition of bands in the preparation from *E. coli* harboring nonrecombinant plasmid was evident (result not shown). SA I/II was recognized by all 22 subjects. Fragment 1 was recognized by 20 of 22, fragment 2 was recognized by 10 of 22, and fragment 3 was recognized by 18 by 22, but fragment 4 was recognized by only 1 of 22 subjects. The responses were therefore directed predominantly towards fragments 1 and 3, although significant responses to fragment 2 were also evident. The frequency of recognition of the truncated form of fragment 3 was also high (16 of 22 subjects). Of those individuals recognizing fragment 3, most (75%) also recognized the truncated fragment.

Since fragment 3 was an immunodominant region with regard to T-cell and B-cell recognition and is involved in binding to salivary receptors (37), B-cell epitopes were mapped by ELISA with the panel of synthetic peptides. The highest dilution of serum which resulted in A_{405} of >0.2 for each peptide was regarded as the endpoint of the titration. Titers which were greater than the mean titer + 2 SD of reactivity with the irrelevant control peptide were considered to indicate recognition. The panel of peptides was screened with sera from 22 individuals, and eight peptides which were recognized by more

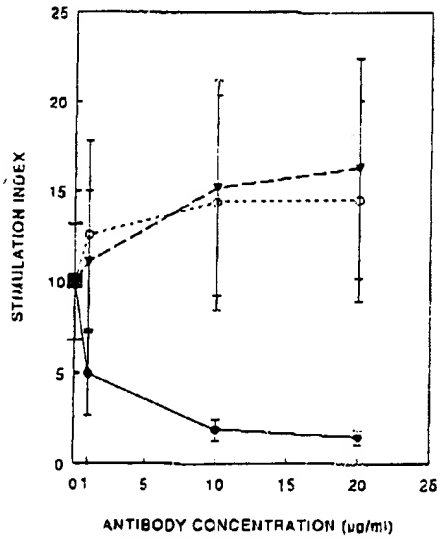

FIG. 2. HLA-DR dependency of proliferative responses to SA I/II. PBMCs were cultured with SA I/II and varying concentrations of MAb L243 (●—●), W6/32 (○----○), or ID4 (▼----▼) (isotype control). Mean SI (± SEM) values are from three subjects.

TABLE 2. Relationship between HLA-DR1 to -6 and the T-cell responses to seven synthetic peptides

DR	SI for peptide[a]						
	803-822	975-994	985-1004	1005-1024	1015-1034	1085-1104	1115-1134
1	**4.1 ± 1.0**	4.0 ± 1.3	**5.8 ± 1.8**	**3.2 ± 0.6**	**3.3 ± 1.1**	**3.3 ± 1.3**	**3.2 ± 0.6**
2	**19.3 ± 6.6**	2.2 ± 0.4	**16.7 ± 1.7**	**14.6 ± 5.7**	**11.2 ± 5.2**	0.6 ± 0.3	**14.7 ± 3.3**
3	**6.1 ± 2.7**	0.7 ± 0.2	**4.1 ± 2.3**	1.0 ± 0.2	2.1 ± 1.7	**4.3 ± 1.2**	**1.9 ± 2.3**
4	**2.5 ± 0.8**	1.8 ± 0.7	**3.0 ± 0.3**	**3.2 ± 0.5**	1.6 ± 0.1	**3.7 ± 0.6**	**1.5 ± 0.7**
5	**6.8 ± 1.0**	1.8 ± 1.3	**2.0 ± 0.8**	**2.3 ± 0.5**	1.3 ± 0.3	1.2 ± 0.4	**2.9 ± 2.8**
6	**2.6 ± 1.5**	**2.9 ± 0.9**	**3.5 ± 0.5**	**8.3 ± 3.1**	**5.7 ± 2.4**	**5.6 ± 1.4**	**5.0 ± 2.0**

[a] SI (±SEM) values of subjects homozygous for DR are shown. Positive responses (SI, >3.0; >500 cpm) are in boldface.

than one individual, together with one peptide which was not recognized, were selected for further analyses (Fig. 4). SA I/II was recognized by all subjects with mean $\log_2$ titer of 7.6 ± 1.2. Titers against the peptides were lower, with only that against peptide 824-843 (mean $\log_2$ titer, 4.7 ± 1.1) being significantly greater than the titer against the control simian immunodeficiency virus p27 peptide ($t = 7.28$, $P < 0.01$). The proportion of significant titers (greater than mean + 2 SD) was also calculated (Fig. 4), and only peptide 824-843 showed high frequency (18 of 22). Indeed, an immunodominant B-cell epitope is present within peptide 824-843, possibly shared with the overlapping peptide 834-853. Peptides 886-905, 925-944, 1035-1054, and 1085-1104 may constitute minor B-cell epitopes since they were recognized with a frequency greater than that of the control peptide but by only a small proportion of subjects (<33%). The sequences of peptides 886-905 and 925-944 are identical. Despite the high frequency of responses to the recombinant polypeptide 984-1161 (described above), a very low frequency of responses was observed to peptides within this region.

Saliva samples from the subjects were cultured to determine levels of *S. mutans*. In 66% of individuals, *S. mutans* was detected (range, 10^3 to 10^5 CFU/ml). There was no correlation between *S. mutans* levels and recognition of particular epitopes or titer against SA I/II.

Adhesion epitope mapping. Adherence of *S. mutans* to saliva-coated microtiter wells was determined with [^{3}H]thymidine-labelled *S. mutans*. The proportion of adhering bacteria was in the range of 1 to 5%, in good agreement with other microtiter plate adhesion assays (47). In the absence of saliva, the proportion of adhering bacteria was <0.1%. In competitive inhibition assays, SA I/II fragments 1 and 3 and the recombinant polypeptide 984-1161 were added to the microtiter wells and incubated with labelled *S. mutans*. SA I/II inhibited adhesion of *S. mutans* in a dose-dependent manner (Fig. 5A) with maximal inhibition of 95% at a concentration of approximately 5.0 μM. Fragment 3 showed maximal inhibition of approximately 60% (at 2.3 μM); fragment 1 did not inhibit, in agreement with previous observations with saliva-coated hydroxyapatite (37). The recombinant polypeptide 984-1161 also inhibited adhesion although less effectively than fragment 3, with maximum inhibition of approximately 40% (Fig. 5A). Myoglobin, a control protein with a similar M_r, did not inhibit adhesion.

The panel of synthetic peptides was then assayed for inhibition of adhesion of *S. mutans* to saliva-coated microtiter wells. Peptides 1005-1024, 1025-1044, and 1085-1104 consistently inhibited adhesion with maximal inhibition of ≥90% at concentrations of 500 μM (Fig. 5B). Adjacent peptides 1015-1034 and 1095-1114 showed more variable and lower inhibition and may be part of the adhesion epitopes.

Caries index and epitope recognition. A group of 24 subjects was divided arbitrarily into a low-caries (DMFS value, 0 to 6) and a high-caries group (DMFS value, >6) (Table 3). The mean T-cell response of the low-caries group ($n = 10$) to the immunodominant T-cell epitope or epitopes within residues 975 to 1004 was SI of 7.93 ± 1.5, which was significantly higher ($P < 0.01$) than the high-caries group ($n = 14$), with a mean SI of 4.08 ± 0.6. The other dominant T-cell epitope(s) (residues 803 to 822) showed no significant difference in the T-cell responses between the low- and high-caries groups. Antibodies to recombinant fragments or to peptides 824 to 843 showed no significant difference in titer between the two caries groups.

DISCUSSION

In this investigation, a fragment of SA I/II (residues 616 to 1213) which inhibits adhesion of *S. mutans* to salivary receptors (37) also expresses immunodominant T- and B-cell epitopes. B-cell recognition was analyzed by ELISA and by Western blotting with recombinant polypeptide fragments and human sera. Although of lower resolution than epitope scanning with synthetic peptides, this approach allows recognition of discontinuous epitopes which would not be represented in a panel of synthetic peptides. The recombinant polypeptide fragments were either not recognized in ELISA or were recognized at lower titers than SA I/II. Fourier transform infrared and circular dichroism spectroscopy indicates that fragments 1 to 3

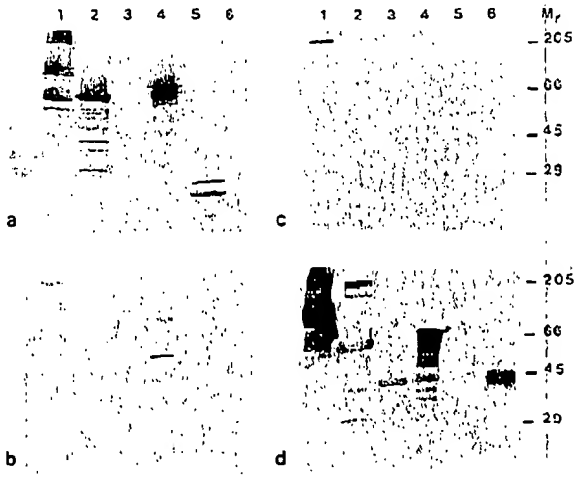

FIG. 3. Serum recognition of SA I/II and recombinant polypeptide fragments. Western blots from three subjects are shown (a to c) together with rabbit anti-SA I/II antiserum (d). Lanes 1, SA I/II; lanes 2, fragment 1; lanes 3, fragment 2; lanes 4, fragment 3; lanes 5, recombinant 984-1161; lanes 6, fragment 4. M_rs (10^3) are shown. Human serum was used at a 1 in 20 dilution.

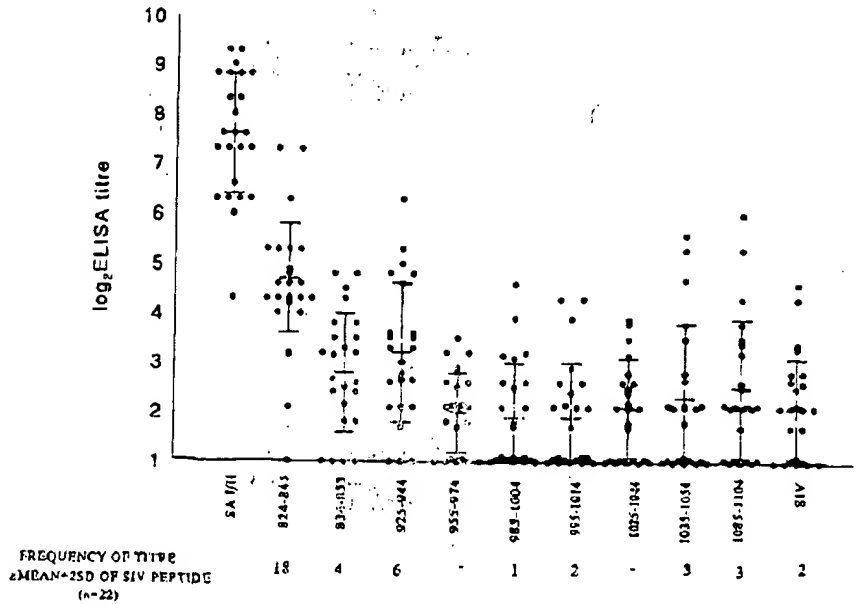

FIG. 4. Human serum recognition of synthetic peptides of SA I/II. Titers to selected peptides of SA I/II and an irrelevant control peptide from simian immunodeficiency virus p27 (SIV) were determined by ELISA for 22 subjects. The frequencies of sera binding the peptides with a titre greater than the mean + 2 SD of the control peptide are also indicated.

possess extensive secondary structure although fragment 3 also included some aggregated or unfolded structure (unpublished data). Thus although the weaker recognition of the fragments may result from incomplete folding, it may also reflect the presence within SA I/II of epitopes comprising residues from more than one fragment. Fragment 3 represents an immunodominant region with regard to recognition by human sera both in ELISA and in Western blotting. The increased frequency with which fragment 1 was recognized by Western blotting compared with ELISA suggests that serum antibodies are induced to epitopes which become accessible only on unfolding of the polypeptide chain. In naturally sensitized humans, such antibodies might be induced to proteolytic fragments of SA I/II. The physiological significance of such antibodies is not clear. Accessibility to antibody may be the major factor in determining antigenicity (1), and in this respect, the antigenicity of fragment 3 is consistent with the proposal that it forms part of a cell wall-distal domain (21).

Epitope scanning of fragment 3 by using the panel of overlapping synthetic peptides (Fig. 6) identified an immunodominant B-cell epitope within peptide 824-843 which represents the N-terminal portion of the proline-rich tandem repeats. An epitope which may overlap with this peptide was also identified in an independent study (34), by using peptides synthesized by the Geysen procedure. Surprisingly, in view of the polar nature of the proline-rich sequences and the likelihood that this portion of SA I/II will adopt an extended conformation (54), only three minor B-cell epitopes were identified in this region (residues 834 to 853 and identical epitopes in peptides 886-905 and 925-944). However, there may be discontinuous epitopes which are not detected by screening with peptides.

T-cell responses of naturally sensitized humans to SA I/II are directed predominantly to residues 816 to 1213 (fragment 3). Immunodominance of this region may reflect intrinsic properties of SA I/II (e.g., structure and antigen processing) or may reflect sequence conservation within SA I/II homologs from other species of streptococci. Fragment 3 represents the most conserved portion of SA I/II (21, 31), and thus raised T-cell and antibody responses may reflect stimulation with cross-reactive antigens from several different species of oral streptococci. Proliferation to SA I/II was completely inhibited by anti-HLA-DR MAb, indicating that the response to SA I/II is HLA-DR restricted although restriction of peptide responses by HLA-DP or -DQ cannot be ruled out. Within fragment 3, two sequences which include promiscuous immunodominant T-cell epitopes were identified: peptides 803-822, which flanks the amino-terminal portion, and 985-1004, which flanks the carboxy-terminal portion of the proline-rich repeats. Both peptides have at least one of the anchor sequence motifs proposed for promiscuous MHC class II-binding peptides (16, 17), whereas the proline-rich sequence includes no such motif, which may account for the lack of T-cell responses to the latter. Several peptides (including those in Table 2) showed promiscuity with regard to HLA class II binding and presentation to T cells. This appears to be a common finding which has been reported previously (5, 7, 41, 48) and was suggested to reflect the requirement for the limited set of different MHC molecules within an individual to bind a large number of diverse peptides (17).

The potential effects of antibodies on the presentation of T-cell epitopes to T cells must be considered. In vitro studies indicate that, during antigen processing, antibody may protect relatively large fragments of antigen from degradation (11, 33). Since these fragments may be unable to associate with MHC class II proteins, the T-cell response is directed away from the protected B-cell epitopes (55). In contrast, overlapping B- and T-cell epitopes have been demonstrated in mice infected with influenza virus (2, 3) and in naturally sensitized humans responding to a streptococcal antigen (8). In this study, convergence of B- and T-cell repertoires may occur within fragment 3, particularly where adjacent immunodominant B-cell (peptide 824-843) and T-cell (peptide 803-822) epitopes were iden-

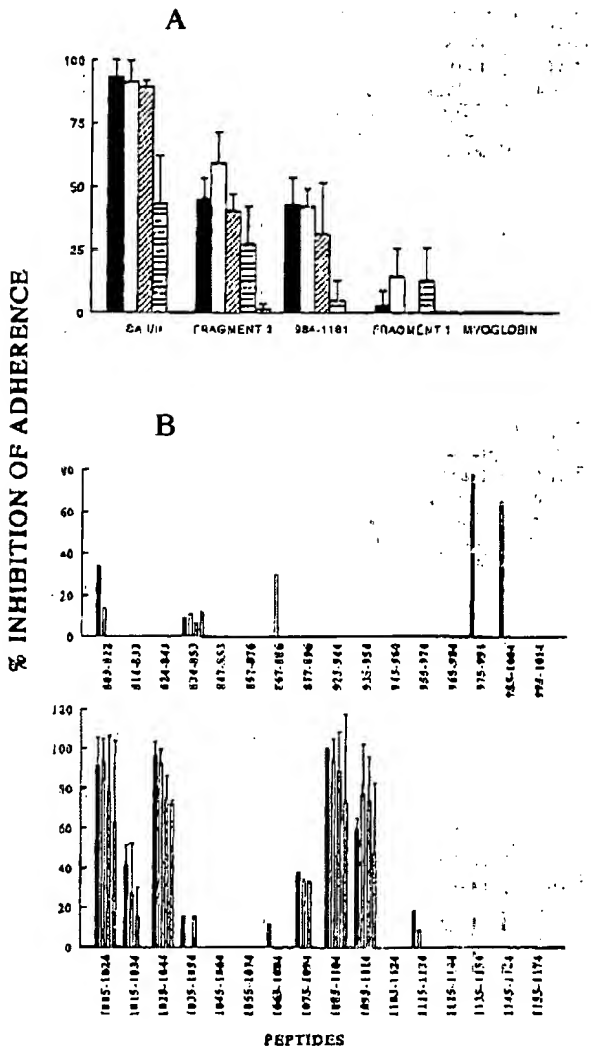

FIG. 5. Inhibition of adhesion of *S. mutans*. (A) Concentrations of inhibitors were as follows: SA I/II, 5.4 μM (■), 2.7 μM (□), 1.35 μM (▨), and 0.675 μM (▭); fragment 3, 4.6 μM (■), 2.3 μM (□), 1.15 μM (▨), and 0.575 μM (▭); recombinant polypeptide 984-1161 and myoglobin (used as a negative control), 5.2 μM (■), 2.6 μM (□), 1.3 μM (▨), and 0.65 μM (▭); fragment 1, 4.2 μM (■), 2.1 μM (□), 1.05 μM (▨), and 0.525 μM (▭). Results (mean + SD) are from three assays. (B) Peptides were assayed for inhibition at concentrations of 500 μM (■), 250 μM (□), 125 μM (▨), and 62.5 μM (▭). Results are from three assays; mean + SD values are shown only for peptides which gave dose-dependent inhibition in each assay.

tified (Fig. 6). An important factor in determining which effect of antibody predominates may be the size of fragment protected from degradation during processing. This may be determined in part by the conformation of the B-cell epitope.

With the objective of determining the adhesion epitope, we have used a novel assay of *S. mutans* adhesion to salivary receptors. Saliva was adsorbed directly to the surface of microtiter wells rather than to hydroxyapatite as described previously (9). This procedure facilitates screening of large numbers of potential inhibitors of adhesion, and although the nature of the adsorbed components may differ from those adsorbed to hydroxyapatite, adhesion of *S. mutans* is SA I/II

TABLE 3. Caries experience and T-cell responses to immunodominant epitopes of SA I/II[a]

Subject group	DMFS value	SI for peptide:	
		975-1004	803-822
High caries (n = 14)	17.5 ± 3.8	4.08 ± 0.6	5.37 ± 0.9
Low caries (n = 10)	2.2 ± 0.7	7.93 ± 1.5	4.2 ± 0.9

[a] Proliferative responses to residues 975 to 1004 and 803 to 822 are shown as mean SI (± SEM). Subjects with a DMFS value of >6 were arbitrarily assigned to the high-caries group, and those with a DMFS value of ≤6 were assigned to the low-caries group; mean DMFS values (± SEM) for each group are shown. Mean ages of the high-caries and low-caries groups were 32.5 ± 2.3 and 28.5 ± 2.1 years, respectively. Counts per minute with medium only were 689 ± 149.

dependent. The specific inhibition observed with SA I/II and fragment 3 but not fragment 1 is in agreement with our previous data with saliva-coated hydroxyapatite as is the observation that SA I/II inhibited more efficiently than fragment 3 (37). We have suggested previously that this may reflect the presence of more than one receptor-binding region in the intact SA I/II and that the receptor-binding region in the alanine-rich sequence (10) may be masked in fragment 1 (37). The lower inhibition mediated by fragment 3 may also be due to incomplete folding of the recombinant polypeptide. A similar consideration may apply to the recombinant polypeptide 984-1161, which inhibited adhesion of *S. mutans* to salivary receptors but to a lesser extent than fragment 3. For the SA I/II homolog (termed SSP-5) from *Streptococcus gordonii*, a calcium-binding region has been identified within the sequence corresponding to residues 1242 to 1324 of SA I/II (14). Thus, the adhesion-binding and calcium-binding sites of SA I/II may be relatively close to each other.

Four peptides, 1005-1024 (NNNDVNIDRTLVAKQSVV KF), 1025-1044 (QLKTADLPAGRDETTSFVLV), 1085-1104 (LATFNADLTKSVATIYPTVV), and the overlapping 1095-1114 also inhibited adhesion (Fig. 6). Amino sugars or other compounds containing primary amino groups can inhibit adhesion in vitro (20, 44). The data presented here, however, suggest that only some of the peptides inhibit adhesion and appear to be specific to *S. mutans*. Moreover, inhibition did not correlate with the number of side-chain amino groups in the peptides. The high concentrations of peptide required for inhibition suggest that adhesion is not mediated simply by short linear sequences of amino acid residues. We suggest that each of the inhibitory peptides includes residues which are essential for adhesion mediated by intact SA I/II. Folding of the native molecule may bring these residues together to form one or more adhesion binding sites. Inhibition of binding of *S. mutans* to salivary receptors has also been described with a 17-residue peptide from the N-terminal alanine-rich region of SA I/II (36), which suggests that there are at least two sites of interaction with salivary receptors.

The finding that there were only minor B-cell epitopes (residues 1035 to 1054 and 1085 to 1104) which may overlap with the two adhesion epitopes within residues 1005 to 1044 and 1085 to 1104, respectively (Fig. 6) is consistent with the success of *S. mutans* in evading antibodies and allowing colonization of teeth. The immunodominant B-cell epitope(s) within residues 824 to 843 is well separated in the sequence from detectable linear adhesion epitopes and is in a portion of SA I/II which does not appear to be essential for adhesion of *S. mutans*. In the sequences of both adhesion epitopes, at least 50% of the residues are polar, suggesting that some portion may be surface exposed. Moreover, interaction with salivary receptors requires these regions to be accessible, and thus the lack of

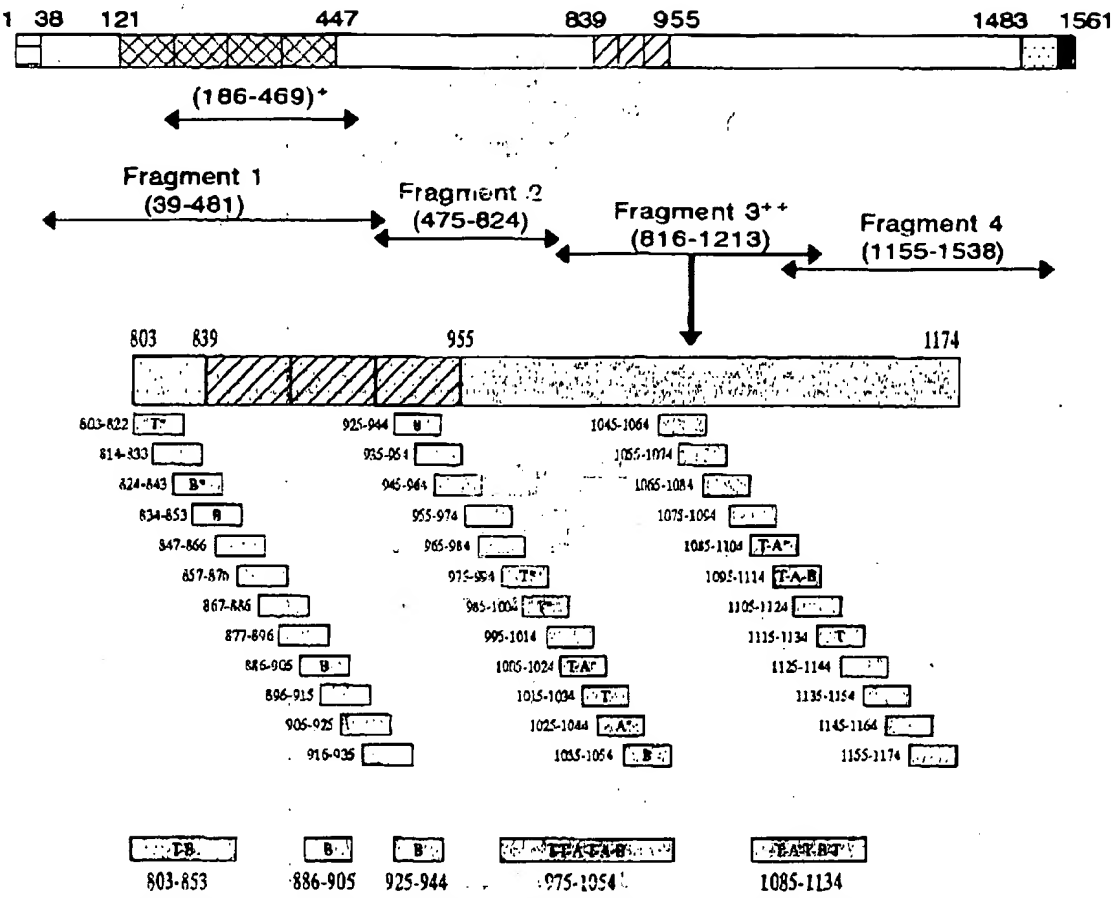

FIG. 6. Mapping of adhesion (A), T-cell, and B-cell epitopes in SA I/II. The main features of the sequence of SA I/II are shown together with the positions of the recombinant fragments (with residue numbers). Symbols: ▯, signal sequence; ▯, alanine-rich repeats; ▯, proline-rich repeats; ▯, cell wall-spanning region; ■, C-terminal hydrophobic sequence. Polypeptides which inhibit adhesion in vitro identified in previous studies are shown: ++, residues 816 to 1213 (37); +, residues 186 to 469 (10). Synthetic peptides (20-mers) spanning residues 803 to 1174 of SA I/II are represented. Those in which T-cell, adhesion, or B-cell epitopes were identified are indicated. Those marked with an asterisk represent dominant epitopes. The three regions which comprise T- and B-cell or T-cell, adhesion, and B-cell epitopes are shown.

B-cell responses to the adhesion epitopes is unlikely to be due to their not being exposed on the surface of SA I/II. An alternative explanation for the limited B-cell response by the adhesion epitopes may be provided by the observation that T-cell epitopes within residues 1005 to 1034 and 1085 to 1104 may overlap the adhesion epitopes. Thus, the peptide overlapping T-cell and adhesion epitopes may lead to silencing of the B-cell responses (29, 46) on account of intracellular competition between the B-cell antigen receptor and MHC class II molecules for the shared epitope. Such competition may prevent or diminish antigen presentation by the B cell and the corresponding T-helper cell (46). It was suggested that, for pathogens, there may be selective pressure for dominant T-cell epitopes to overlap critical determinants and thereby block antibody responses to those determinants (46).

Mapping of the T- and B-cell and adhesion epitopes gives a linear, though not spatial, relationship between the major T-cell–adhesion–B-cell epitopes (Fig. 6). This shows up to seven T-cell, four B-cell, and four adhesion epitopes. As some of the epitopes are overlapping, or they are adjacent or in proximity, and most of these are likely to be discontinuous, the total number of T- and B-cell and adhesion epitopes will be smaller. There are clearly three spatially separated polypeptides of which the most amino-terminal polypeptide, 803-853, expresses a major T-cell and B-cell epitope. The second polypeptide (residues 975 to 1054) appears to be the most significant, as it contains one major and two to three minor T-cell, one major and one minor adhesion, and one minor B-cell epitope (Fig. 6). The third polypeptide (1085-1134) expresses two to three minor T-cell epitopes, a major adhesion epitope, and a minor B-cell epitope (Fig. 6). Although only analysis of a crystal structure will yield the three-dimensional structure of the SA I/II fragment 3 protein, the epitope map offers some insight into the subtle relationship between microbial adhesion and its evasion of the host's T- and B-cell responses.

A recombinant polypeptide comprising the immunodominant T-cell epitope and adjacent adhesion epitopes (residues 975 to 1044) was prepared and used to immunize mice. This peptide induced T-cell responses to peptide 985-1004 and antibody responses to peptides 995-1015 and 1025-1044, within which one adhesion epitope resides (unpublished data). Thus by immunizing with appropriate constructs of T-cell and adhesion or T-cell, adhesion, and B-cell epitopes, antibody responses to adhesion epitopes can be induced. Such constructs

might form the basis of a subunit vaccine against dental caries. An effective vaccine must induce responses only to *S. mutans* and not to noncariogenic commensals. In this respect, the number of substitutions in the adhesion-mediating sequences (i.e., residues 1005 to 1044 and 1085 to 1114) compared with the homologous regions of *S. gordonii* (total of 17 substitutions) and *Streptococcus sobrinus* (total of 12 substitutions) may allow species-specific responses. These substitutions may also influence the adhesion receptor specificity of these microorganisms. Similarly, the number of substitutions within residues 975 to 1004, which include one or more immunodominant T-cell epitopes, compared with *S. gordonii* (10 substitutions) and *S. sobrinus* (12 substitutions), may result in species-specific T-cell responses. This, however, remains to be determined.

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